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(54) Title: LIVE RECOMBINANT VACCINE COMPRISING INEFFICIENTLY OR NON-REPLICATING VIRUS

(57) Abstract

The subject invention pertains to a novel recombinant vaccinia virus vaccine for use in immunizing animals and humans against disease. The vaccine comprises a live vaccinia or replication deficient mutant vaccinia virus capable of expressing a single or multiple heterologous genes or gene fragments. In a preferred embodiment, the recombinant virus is contained in an orally-administered package that will only dissolve in the host animal's gut. The subject invention also pertains to a method of inducing a broad protective immune response through the oral, intranasal, or other mucosal means of administration of the recombinant vaccinia virus vaccine.

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Live Recombinant vaccine comprising inefficiently or non-replicating virus.

Background of the Invention

The subject invention described herein relates to the field of vaccines. Each year millions of children die of vaccine-preventable diseases. It is estimated that at least one child in five, including many of the children in the United States under the age of 2, have not been fully vaccinated (Gibbons, 1994). To help solve this problem, The Children's Vaccine Initiative proposed the development of a multivalent, heat-stable, inexpensive, orally-administered, safe and effective vaccine. Oral administration of vaccines may also help reduce children's fear of shots at the doctor's office.

A vaccine based on recombinant vaccinia virus meets many of these requirements. Vaccinia is a non-oncogenic virus that reproduces entirely within the cytoplasm of a host eukaryotic cell. Methods for inserting foreign DNA sequences into vaccinia are known in the art and at least 25 kb of heterologous DNA (representing about 10-20 genes) can be inserted into vaccinia virus genome (Smith *et al.*, 1983). When administered parenterally, an immune response can be induced to the products of multiple heterologous genes expressed by a recombinant virus (Perkus *et al.*, 1985). Lyophilized vaccinia virus is very heat-stable, maintaining infectivity after two hours at 100°C (Arita, 1973). Vaccinia vaccine is also inexpensive, having been produced for about $.03 to $.04/per dose during the smallpox eradication program (Fenner *et al.*, 1988). In addition, vaccinia virus can be readily grown in laboratory cultures and prepared in stable, freeze-dried forms.

Orally-administered liquid vaccinia/rabies recombinant vaccines have been used to successfully immunize animals in the wild (Blancou *et al.*, 1986 and Rupprecht *et al.*, 1986), probably as a result of viral replication in the tonsils (Rupprecht *et al.*, 1988). However, there are several problems associated with oral administration of a liquid containing vaccinia or vaccinia recombinants. It can produce oral lesions (Greer *et al.*, 1974 and Hochstein-Mintzel *et al.*, 1972) and has been shown to be poorly immunogenic in chimpanzees when given orally (Rupprecht *et al.*, 1992). In addition, the vaccinia virus is rapidly destroyed by stomach acid or bile (Hochstein-Mintzel *et al.*, 1972), thereby eliminating intragastric immunization as a viable mode of administration.[HAC1] In addition, because vaccinia can replicate in the eukaryotic cell cytoplasm, the potential for replication of a pathogenic virus vector cannot be completely ruled out when using standard vaccinia virus as a recombinant vector for vaccine purposes. It is for this reason that in a preferred embodiment of the present invention, a

replication deficient virus is used as the vector.

The effectiveness of a particular vaccine may depend upon which cells or tissues of the immune system are activated by the vaccine. The role of three arms of the immune system, namely serum antibody, mucosal IgA antibody, and cytotoxic T-lymphocyte (CTL) activity, have been studied using an influenza viral challenge of mice. In this model, secretory IgA antibody has been shown to prevent infection of the nose (Renegar *et al.*, 1991a; Renegar *et al.*, 1991b), and serum IgG antibody has been shown to prevent infection of the lung (Loosli *et al.*, 1953). When the virus evades these protective antibodies, anti-influenza CTL activity enhances recovery (Yap *et al.*, 1978, Lin *et al.*, 1981, and Wells *et al*, 1981).

Intradermal (scarification) administration of a recombinant vaccinia virus that expresses the influenza hemagglutinin protein (vac/H1) has been shown to induce both serum antibody that prevents viral pneumonia (Smith *et al.*, 1983, Small *et al.*, 1985, and Bennink *et al.*, 1984) and CTL activity that promotes recovery (Bennink *et al.*, 1984, Andrew *et al.*, 1987, and Bender *et al.*, 1990). However, this route of vaccine administration does not induce mucosal IgA antibody (Small *et al.*, 1985 and Meitin *et al.*, 1993). Intranasal, intradermal and enteric immunization of cotton rats with a live recombinant vaccinia virus expressing the respiratory syncytial virus F glycoprotein has also been reported (Kanesaki *et al.*, 1991). Intranasal administration of vac/H1 recombinant virus induces serum IgG antibody, IgA antibody in nasal wash and CTL activity (Meitin *et al.*, 1991); however, there are several practical limitations to this approach with humans. For example, storage constraints, temperature stability, and intranasal administration of liquids may pose practical problems in developing countries.

Thus, there remains a need for a vaccine that is temperature-stable, inexpensive, easily administered and which also induces serum IgG, mucosal IgA and cell-mediated immune responses. Such a recombinant vaccine should meet all the requirements of The Children's Vaccine Initiative and thereby greatly facilitate immunization, especially in developing countries.

Brief Summary of the Invention

The subject invention concerns a novel recombinant vaccine composition for immunizing animals, including humans, against pathogenic disease organisms. Specifically, the vaccine comprises a live vaccinia, mutant vaccinia virus, such as a replication deficient or

a highly attenuated vaccinia of which modified vaccinia virus Ankara (MVA) is a preferred virus, deletion or insertion mutants of vaccinia virus, or a canary pox virus, (each of which is referred to herein generally as "vaccinia" for ease of reference), that expresses a heterologous gene or genes. In a preferred embodiment, the recombinant virus is contained in an orally-administered, enteric-coated capsule or other suitable dosage form that will only dissolve and release virus when it reaches the host's small intestine. Once in the intestine, the recombinant virus induces a host immune response against the expression product of the heterologous genes. Advantageously, such an administration of the recombinant vaccine according to the subject invention is capable of inducing serum IgG, mucosal IgA and a cell-mediated immune response by the host animal. In another preferred embodiment, a recombinant, replication deficient virus is provided in an intranasal dosage form. In a further embodiment of the invention, a recombinant, replication deficient viral vector is used to induce immune responses in a geriatric mammal. In a further embodiment, a recombinant, replication deficient viral vector is used to induce mucosal, serum and cellular immune responses against an antigen in large mammals, including horses, pigs, goats, sheep, cows, and primates, including humans. Thus, the vaccine of the subject invention is capable of providing multiple levels of immune protection against pathogenic infections in a form that is inexpensive, environmentally stable, easily administered, safe and effective.

The subject invention further concerns a method of inducing a protective immune response by immunization with an orally administered live recombinant vaccinia virus. The induced serum IgG, mucosal IgA and cell-mediated responses are directed against the heterologous gene product(s) expressed by the recombinant virus. The multi-level immune response induced by the subject vaccine confers protective immunity on a host from targeted pathogens.

In one embodiment of the subject invention, the enteric administration of a recombinant vaccinia virus that expresses the influenza hemagglutinin gene (this recombinant virus is referred to herein as vac/H1) induced mucosal IgA and serum IgG anti-H1 antibody, in addition to inducing CTL activity in mice. This immune response provided protection of both the nose and lungs of the mice from a subsequent viral challenge with influenza.

Enteric immunization with MVA and recombinant MVA is very safe; neither MVA nor MVA containing the influenza virus H1 and NP genes (MVA HA-NP) killed any SCID (B- and T-cell deficient) mice. In addition, we have discovered that three doses of MVA(H1+NP) induce high levels of immunity in a mouse model. The titers persisted for at

least 52 weeks (half the life-span of the mouse) and the noses and lungs of the immunized mice were still protected from an influenza challenge at 52 weeks. In a further embodiment, we found that 3 doses of intragastric MVA(H1+NP) induced anti-influenza serum IgG antibody and mucosal IgA antibody in aged (22-24-month-old) mice, even though the levels were about 5-fold lower in the aged animals. The vaccine induced neutralizing antibody in both groups of animals. In yet a further embodiment, we have found that higher mammals, when immunized with this vaccine, exhibit elevated neutralizing immune responses, indicating efficacy and important benefits of this vaccine for both the veterinary as well as human recipients, and in particular for geriatric recipients. MVA (H1+NP) given intranasally in an equine model induces serum and mucosal influenza neutralizing antibody, and blood from immunized horses gives approximately a 3 log reduction in the *in vitro* growth of MVA(H1+NP). Furthermore, we have established that MVA(H1+NP) cannot be isolated from the blood of inoculated horses. We have further established that MVA(H1+NP) cannot be isolated from horse feces following intrajejunal inoculation. In addition, we have found that MVA(H1+NP) cannot be recovered from nasal secretions following intranasal inoculation.

Brief Description of Drawings

Figure 1 shows influenza virus titers from the nose (upper graph) and lungs (lower graph) of research animals. Titers are reported as the $\log_{10}$ of the 50% egg infectious doses (log EID_{50}). All points represent the average of 6 animals except intragastric responders and intragastric-nonresponders: on days 1, 3, and 7, there were four animals in the responder group and two animals in the nonresponder group; on day 5 there were three animals in each group. Undetectable virus was defined as 0 on this log scale.

Figure 2 shows the induction of anti-H1 influenza virus specific serum antibodies by various vaccines or control. Mice were given 10^8 pfu of MVA HA-NP by two intragastric (i.g.) inoculations (□). Control mice were inoculated intranasally (i.n.) with influenza A/Puerto Rico/8/34 (h); i.m. with MVA (Î) or MVA HA-NP (O); or were naive (~). Serum was obtain from tail vein and vaginal wash fluid was obtained by flushing the vagina 6-8 times with the same 80 :l of PBS (Meitin, *et al.*, 1994) during weeks 2, 4, and 8. The ELISA to measure anti-H1 serum antibodies was performed as previously described (Meitin *et al.*, 1991) and data points are plotted as X 10^{-3}. Vaginal wash samples were frozen at -20E C and later tested in an ELISA for anti-H1 IgA antibodies. To dissolve mucous strands, an equal

quantity of 0.01 M dithiothreitol (Sigma, St. Louis, MO) was added and the samples vortexed, incubated at room temperature for 30 minutes, and centrifuged for one minute at 15,000 X g. Duplicate two-fold dilutions of samples were run in wells precoated with influenza hemagglutinin and in wells not containing antigen followed by goat anti-mouse igA (Sigma). The labeling reagent was alkaline phosphatase linked to rabbit anti-goat IgG (ICN Immunobiologicals, Irvine, CA) and p-nitrophenyl phosphate (Sigma) was used as substrate. Color was allowed to develop for 45 minutes at room temperature and absorbance at 405 nm was read on a Titertek Multiscan (Flow). Titers are expressed as the highest dilution for which the OD of the positive (antigen-containing) well divided by the OD of the respective negative (control) well gave a ratio greater than or equal to 2. The results are plotted as the geometric mean titer of the ELISA values (n=5-6/group) versus time after the first inoculation.

Figure 3 shows nasal (A) and pulmonary (B) influenza virus titers *in vivo* following virulent homologous influenza virus challenge of animals pretreated with various vaccines or control. Twenty four days after the second inoculation with MVA HA-NP, mice were challenged with H1N1 and sacrificed one (closed symbols) and three (open symbols) days later. The data are a summary of two experiments challenging mice with using $10^{7.0}$ $TCID_{50}$ (•) and $10^{4.1}$ $TCID_{50}$ (□, O) of influenza virus. Virus titers were virtually identical for naive or i.m. MVA mice and are not shown. Day one nasal viral titers were significantly lower than those of control mice (naive and MVA i.m.) for MVA HA-NP i.g. ($P<0.001$, Exp. #1; $p<0.05$, Exp. #2) and H1N1 recovered ($p<0.001$, Exp. #1; $p<0.05$, Exp. #2), but not for MVA HA-NP i.m. ($p>0.05$, Exp. #1 and #2). Day one pulmonary viral titers were significantly lower than control for MVA HA-NP i.g. ($p<0.001$, Exp. #1 and #2), H1N1 recovered ($p<0.001$, Exp. #1 and #2), and MVA HA-NP i.m. ($p<0.001$, Exp. #1 and #2). Data were analyzed using InStat 2.00 (GraphPadSoftware, San Diego, CA) and a Power Macintosh 6100/66 computer. A one way ANOVA was followed by Student-Newman-Keuls multiple comparisons test.

Figure 4 shows mean nasal (A) and pulmonary (B) viral titer *in vivo* 5 days following virulent heterologous influenza virus (H3N2) challenge of animals pre-treated with various vaccines or control. Four weeks after the second inoculation with MVA HA-NP, mice were challenged with H3N2 and sacrificed one and five days later. Day one titers (not shown) varied from 1.8 to 2.5 $TCID_{50}$ in the nose and 3.0 to 3.6 $TCID_{50}$ in the lungs and were not significantly different between groups. The data are a combination of two separate experiments using $10^{6.5}$ (•) and $10^{4.6}$ (□) $TCID_{50}$ as challenge inoculation. Day 5 nasal viral

titers were significantly lower than control (naive and MVA i.m.) for MVA HA-NP i.g. ($p<0.05$, Exp. #1 and #2) and H1N1 recovered ($p<0.01$. Exp. #1 and #2). but not MVA HA-NP i.m. ($p>0.05$, Exp. #1 and #2). Day 5 pulmonary viral titers were significantly lower than control for MVA HA-NP i.g. ($p<0.05$. Exp. #1 and #2 X1 or X2 or both). H1N1 recovered ($p<0.001$. Exp #1 and #2). and MVA HA-NP i.m. ($p<0.05$. Exp. #1 $p<0.05$. Exp. #2).

Figure 5 shows the results of inoculation of nude mice with recombinant vaccinia viruses is safer by the intragastric than parenteral route.

Figure 6 shows that MVA/H1+NP is not lethal in SCID mice (no deaths post inoculation. top panel: no weight loss. bottom panel). while vaccinia recombinants carrying the influenza HA gene are lethal.

Figure 7 shows that three doses of MVA HA-NP induced long-term serum and mucosal anti-influenza antibody (serum IgG. top panel: mucosal IgA. bottom panel). and since an increased response is achieved between the second and third doses. elicitation of anti-vector (MVA) antibodies does not appear to limit booster of desired immune responses.

Figure 8 shows the comparative elicitation of serum (top panels) and mucosal (bottom panels) immune responses in young (left hand panels) and aged (right hand panels) of mice. showing a reduced but still clearly significant immune response in aged mice.

Figure 9 shows the induction of influenza neutralizing antibodies in young and aged mice.

Figure 10 shows protection of mice challenged with H1N1 virus after no treatment (negative control. in convalescent mice. in mice that received standard vaccine. or MVA-HA-NP orally or intranasally), with the vaccine of this invention providing the greatest protection against weight loss in the challenged animals.

Detailed Description of the Invention

The subject invention concerns novel recombinant vaccinia. mutant vaccinia virus. such as a replication deficient or a highly attenuated vaccinia of which modified vaccinia

virus Ankara (MVA) is a preferred virus, deletion or insertion mutants of vaccinia virus, or a canary pox virus, (each of which is referred to herein generally as "vaccinia" for ease of reference), compositions and methods of inducing a broad immune response in a host organism using this vaccine. Specifically, the subject invention concerns a live recombinant vaccinia virus that can express the products of one or more heterologous genes. When expressed by the vaccinia virus in a host animal these gene products induce a multi-component immune response in the host. The immune response includes serum IgG antibody, mucosal IgA antibody, and cell-mediated responses directed against the heterologous gene products. Thus, once vaccinated according to the subject invention, the host animal is either protected from infection or at least primed to mount a fully protective secondary immune response upon exposure to any pathogen expressing the heterologous gene products to which the host has been sensitized. Accordingly, the host animal vaccinated with the vaccine of the subject invention can be effectively immunized against a wide variety of pathogens including bacteria, viruses, fungi, and parasites.

In an exemplified embodiment, the live recombinant vaccinia virus is administered enterically to a host animal. In another embodiment, a mutant form of a recombinant vaccinia virus, such as a strain that is replication deficient in mammalian cells, can be used according to the subject invention. More preferably, the recombinant virus is orally administered in a form that releases the virus only in the intestine of the host animal. Techniques for preparing live vaccine in enterically-coated dosage forms are known in the art (see, for example, Stickl, A.H., British Patent No. 1-333-512). As referred to herein, the term "intestine" is meant to include both the large and small intestinal tracts. In a preferred embodiment, immunization with the subject vaccine occurs primarily in the small intestine.

Enteric administration of replication competent recombinant virus results in the exposure of IgA precursor B cells in lymphoid tissue of the intestine, such as Peyer's Patches, to the expressed heterologous gene products. These IgA precursor B cells can then migrate to mucosal tissues, such as the respiratory tract, where they may differentiate into mature IgA-secreting plasma cells. In addition, Ig secreting B cells in other lymphoid tissues, and various components of the cellular immune system, such as T cells and macrophage, are stimulated upon exposure to the heterologous gene products.

In another exemplified embodiment, higher mammals are inoculated intranasally with a replication deficient recombinant viral vector, and neutralizing immune responses are thereby induced. Preferably, the recombinant vaccinia virus of the subject invention contains

multiple heterologous genes that encode polypeptide antigens which are expressed after introduction into the host system. A polyvalent vaccine according to the subject invention can be used to immunize a host animal against multiple diseases produced by a variety of pathogenic organisms. Similarly, such a polyvalent vaccine can be used to induce a broad immune response against a single type of pathogen, particularly those pathogens that express various forms of antigens or that express different antigens at different times during their life-cycle.

The recombinant vaccinia virus of the subject invention can be used with a variety of heterologous genes or gene fragments. For example, genes from pathogens that cause influenza, measles, hepatitis B, diphtheria, tetanus, mumps, rubella, and others, can be inserted into the recombinant vaccinia. The subject invention can be used with a wide variety of gene inserts. Thus, the subject invention can be used to prevent diseases, such as pertussis, tuberculosis, cholera, and immune deficiency conditions induced by infection, upon insertion into the vector of appropriate gene inserts. In addition, the subject invention can be readily used in other areas of vaccine technology, such as in cancer prevention or fertility control, upon insertion into the vector of appropriate antigens. The subject invention also contemplates the use of chimeric genes that express a fusion product comprising the expression products of a portion of two or more heterologous genes. The subject invention further contemplates the use of genes that encode protein structures that mimic polysaccharide antigens.

The subject invention can be used in vaccinating both animals and humans against pathogenic organisms. The vaccine can be administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered can depend upon the subject to be treated, the immunogenicity of the expressed gene products, and the degree of protection desired. Dosage parameters can be readily determined by those skilled in the art using the disclosure provided herein.

As described herein, intrajejunal immunization with a recombinant vaccinia expressing the influenza hemagglutinin protein (vac/H1) in a influenza/mouse model system consistently induced nasal, gut, and vaginal wash IgA antibody as well as serum IgG antibody and CTL activity in the host mouse that was directed against the hemagglutinin. The nasal IgA antibody was responsible for a significant reduction in nasal virus following challenge of enterically immunized mice (Renegar *et al.*, 1991a,b). The serum IgG antibody was

responsible for a significant reduction in lung virus following challenge of immunized mice. Mice immunized intrajejunally multiple times with the recombinant vaccinia vac/H1 developed an immune response to the hemagglutinin in spite of having been immunized parenterally fifty-three days earlier with a wild-type (WR) strain of vaccinia.

To be of practical value, a vaccine must be safe for both the vaccinated person and that person's contacts. Mice immunized with the Vac/H1 recombinant showed no ill effects from enteric immunization. The mice had no change in bowel habits or ruffled fur. The enterically immunized mice did not shed vaccinia virus in their feces. However, if other species do excrete vaccinia virus, or if the vaccinia is potentially harmful to the animal, replication deficient mutant forms of vaccinia strains such as MVA (Sutter *et al.*, 1994) or a canary pox virus (Cadoz *et al.*, 1992) can be used.

There are two potential disadvantages with using recombinant vaccinia viruses as human vaccines. The first issue is safety. During the Smallpox Eradication Programme, serious adverse reactions from vaccinia occurred at a rate of 38/1,000,000 for eczema vaccinatum, 1.5/1,000,000 for progressive vaccinia, and 12/1,000,000 for post-vaccinial encephalitis. Because of the risk of dissemination, vaccination was contra-indicated in infants with eczema or anyone suffering from immune dysfunction (Centers for Disease Control, 1991; Fenner, *et al.*, 1988). These risks should be negligible with avian host restricted poxvirus such as canarypox (Cadoz *et al.*, 1992) and replication deficient vaccinia virus such as and including modified vaccinia virus Ankara (MVA). MVA was derived prior to eradication of smallpox from repeated (over 570) passages of vaccinia virus Ankara in chicken embryo fibroblasts (Hochstein-Mintzel, *et al.*, 1972). Genetic analysis revealed that MVA had suffered six major deletions of its genome, resulting in the loss of 30,000 base pairs (15% of its genome) so that it became host-restricted and unable to grow efficiently in mammalian cell lines (Meyer *et al.*, 1991). The block in replication of MVA in human cells occurs at a step in virion assembly rather than at an early stage of infection as happens with some other poxvirus host-restricted mutants (Sutter *et al.*, 1992). MVA was found to be avirulent in both normal and immunocompromised animals and was given to 120,000 people, many at high risk of complications from the standard vaccine, without significant side effects (Hochstein-Mintzel, *et al.*, 1972, *supra*; Werner *et al.*, 1980; Mayr, *et al.*, 1978; Mayr *et al.*, 1975, Mayr *et al.*, 1979; Stickl *et al.*, 1974). Further, recombinant gene expression is unimpaired (Sutter *et al.*, 1992, *supra*). Mice injected intranasally, subcutaneously, intramuscularly or intravenously with a recombinant MVA that expresses the genes of

influenza hemagglutinin (HA) and nucleoprotein (NP) developed anti-H1 serum IgG antibodies and CTL activity and were protected from a lethal challenge with a homologous influenza virus (Sutter *et al.*. 1994). There was no evidence from this report relating to oral immunization using recombinant MVA. and induction of IgA is not demonstrated.

The second issue that lowered enthusiasm for vaccinia vectors was the demonstration that while parenteral immunization stimulated protective levels of serum IgG antibody and cellular immunity, it did not induce mucosal immunity (Small *et al.*, 1985). We have demonstrated. however. that intragastric administration of a recombinant vaccinia virus induces solid mucosal. humoral. and cellular immunity. Moreover. vaccinia/rabies recombinants given as oral bait have successfully immunized animals in the wild. probably as a result of viral replication in the tonsils (Blancou *et al.*, 1986: Rupprecht *et al.*, 1986; Rupprecht *et al.*. 1988).

Non-replicating or replication defective vectors can also include a gene for the expression of interleukin-2 in order to supplement the induced immune response (Flexner *et al.*, 1987). Thus. oral administration of an enteric coated recombinant vaccinia or mutant forms of recombinant vaccinia can provide a safe and effective mode of immunization. The development of multivalent vaccinia recombinants makes the subject invention even more effective.

What is most surprising is that. according to the instant invention. a replication-deficient viral vector. exemplified herein by a recombinant MVA. is capable of inducing significant immune response which in appropriate animal models exhibit significant indicia of efficacy in man and other vertebrates. Those skilled in the art will recognize. based on this disclosure, that the term replication defective means that the virus cannot replicate. or replicates inefficiently, absolute absence of any replication being difficult to confirm.

Our studies clearly demonstrate the potential immunogenicity and efficacy of MVA HA-NP as an oral, enteric or intranasal vaccine. One oral dose of the vaccine induced low levels of both mucosal and serum antibodies. A second i.g. dose of MVA HA-NP significantly boosted the titer of anti-influenza serum antibody and, especially, mucosal IgA antibody showing that the first dose primed the mice for an anamnestic response. The biological effectiveness of these immunizations was shown in a series of challenge experiments. The mice that received one i.g. dose of MVA HA-NP were partially protected from a homotypic challenge and all of those that received two doses were fully protected from pulmonary infection. Most (10 to 17 mice) were also protected from nasal infection. One

i.m. dose of MVA HA-NP completely protected the lungs, but not the noses. These data serve to emphasize the importance of mucosal versus serum antibodies in the prevention of infection of the upper and lower respiratory tract, respectively (Renegar *et al.*, 1991a; Renegar *et al.*, 1991b; Barber *et al.*, 1978; Clements *et al.*, 1986).

Recovery was evaluated and demonstrated herein by data generated in experiments with heterologous, H3N2 influenza virus. These showed that viral clearance of both the lung and the nose was enhanced with i.g. MVA HA-NP, while i.m. MVA HA-NP enhanced recovery of only the lungs (Figure 4). Based on previous studies, this was most likely due to induction of anti-NP CTL activity, but other factors may be involved. For example, we recently showed that heterotypic immunity can be induced in $2m-/-mice; the mechanism of this protection did not involve class I major histocompatibility complex (MHC)-restricted T cells, but may have been due to induction of class II MHC-restricted CD4+T cells or induction of mucosal IgA (Bender *et al.*, 1994; Mazanec *et al.*, 1992; Mazanec *et al.*, 1995). Regardless of the mechanism of the enhanced recovery these experiments highlight the importance of using a conserved protein such as NP (Gammelin *et al.*, 1991) in a vaccine in order to generate a heterotypic protective response.

Oral immunization with replication-deficient recombinant vaccinia virus such as MVA offers many advantages over other vaccine candidates. First, as demonstrated here, this method is not only effective in inducing immune response in all three arms of the immune system)serum antibody, mucosal IgA antibody, and cell-mediated immunity)it is also efficacious. Use of an oral or intranasal rather than a parenteral vaccine may enhance patient (and/or parental) acceptance and would obviate the need for syringes and needles. Second, MVA is an extremely safe vector that has undergone extensive safety testing in humans and animals (Hochstein-Mintzel, *et al.*, 1972; Werner *et al.*, 1980; Mayr, *et al.*, 1978; Mayr *et al.*, 1975, Mayr *et al.*, 1979; Stickl *et al.*, 1974; Meitin *et al.*, 1991). Third, multivalent recombinant MVA can be constructed. Even if it proves to be too difficult to transfer more than a few genes into MVA or have them expressed in appropriate concentrations, a cocktail of recombinant MVA viruses, each containing several genes, would be expected to work based on the instant disclosure. Fourth, lyophilized vaccinia is extremely heat-stable. Heating to 100E C for two hours lead to a loss of only one log of infectivity. After storage at 45E C for two years, it was still 100% successful in vaccination of volunteers (Cross, 1957). These properties make oral recombinant MVA an ideal candidate vaccine to meet all the requirements of the Children's Vaccine Initiative (World Bank, 1993; Bloom, 1989; Robbins

et al., 1988). It could provide children in the developing world with an ideal vaccine and save children throughout the developed world from the fear of shots.

From the foregoing description, it should be apparent that success in using an MVA recombinant for oral immunization could not have been predicted. In contrast to parenteral injection with replication competent or replication deficient viruses, wherein the virus can directly reach cells of the immune system, a replication deficient vaccinia or pox virus introduced into the gut would not be expected to directly reach cells of the immune system. Therefore, *a priori*, it was not known whether immunization with a replication defective vaccinia or pox virus, via the small intestine would be effective. This is because access to immune responsive cells in the intestine is severely restricted, with the likely outcome of such a mode of immunization being abortive infection of intestinal epithelium. Even having demonstrated the effectiveness of this method herein, the mechanism is unknown, although it is postulated that the M-cells covering Peyer's patches in the gut may play a role. In addition, in one embodiment of this invention, particular technical advances have been made which contribute to the success of an oral MVA based vaccine. In particular, the evidence adduced herein of efficacy of the oral vaccine is extremely significant and novel. In addition, we have pre-treated the animal models with an acid release blocker (cimetidine, but any like agent, such as PEPCID may be used) to prevent destruction of the oral vaccine en-route to the intestine. In addition, we pre-treated the animals with an active analog of the cholecystokinin hormone to ensure that bile would not be available to destroy the vaccine. Either or both of these pre-treatments, or modifications thereof may be used with enteric coated virus.

In humans, both of these pre-treatments may be acceptable. However, in terms of the bile secretions, pre-treatment with a cholecystokinin antagonist such as are well known in the art, may be more clinically acceptable. This pre-treatment would ensure that bile is not released when the vaccine is en-route. These pre-treatments may be less important with an efficiently enteric-coated vaccine, but may be of assistance. For the purposes of the present studies, these pre-treatments provided for efficient oral immunization.

Most recently, we have extended our studies in murine models to demonstrate that the vaccine of this invention, given either intragastrically or intranasally, is capable of raising significant protective immune responses in geriatric mice. In addition, we have also been able to show that a recombinant MVA carrying influenza virus genes is capable of inducing significant viral neutralizing immune responses in an equine model. Based on these results, we conclude that a recombinant vaccine according to this invention harboring a gene for any

given antigen may be used to induce serum, cellular and mucosal immune responses upon appropriate administration thereof either to the intestinal or nasal mucosa.

Materials and Methods

Unless indicated otherwise, the following materials and methods were used.

Immunization of animals. Female BALB/c mice, 6-8 weeks old were given a single immunization either by scarification of the tail, intravenous, intranasal, intragastric, or intrajejunal administration of 10^8 pfu of a vaccinia-influenza recombinant containing the hemagglutinin gene from H1N1 PR8 influenza (this recombinant is referred to herein as vac/H1) which was constructed as previously described (Flexner *et al.*, 1987). All mice were fasted overnight before immunization. Intragastric administration was accomplished by use of an oral gastric tube. Intrajejunal administration was done by injection during laparotomy. Animals were anesthetized with 0.1-0.2 ml of sodium pentobarbital (0.09 g/ml) and the peritoneal cavity was entered. The stomach and small bowel were identified and the vaccine was placed into the lumen of the jejunum with a 26 gauge needle. The incision was then closed. The three control groups consisted of naive mice, mice injected intraperitoneally (i.p.) two times with inactivated H1N1 influenza vaccine, and mice infected intranasally while awake with live H1N1 PR8 influenza virus.

Six weeks following inoculation, the mice were sacrificed. Splenic lymphocytes were collected for measurement of anti-influenza cytotoxic T lymphocyte (CTL) activity. Serum, nasal wash (Renegar *et al.*, 1991b), gut wash, and vaginal wash (Wu *et al.*, 1993) were collected for anti-influenza antibody determination. These were later assayed by ELISA for serum IgG as well as nasal and gut wash IgA anti-influenza antibody, and the titers were then calculated by comparison with monoclonal controls (Meitin *et al.*, 1991). Data were analyzed using a one factor ANOVA. Student-Newman-Keuls *post hoc* test was used to analyze differences between groups.

Cytotoxic T Lymphocyte Assay. Spleens were obtained from BALB/c (H-2^d) mice 6-8 weeks post-intrajejunal immunization with 10^8 pfu of vac/H1. Spleens were also obtained from H1N1-infected mice, wild-type vaccinia-immunized mice, and naive mice. Spleen cells were then cultured for seven days with H1N1-sensitized autologous splenocytes and then tested in a 6 hr ^{51}Cr release assay against vac/H1-, vaccinia containing the nucleoprotein gene (vac/NP-), or H1N1-sensitized P815 (H-2^d) mastocytoma cells (Bender *et al.*, 1991). Percent (%) specific lysis was determined as: (experimental release-spontaneous release/total release-

spontaneous release) x 100. Multiplicity of infection was 100 pfu per cell for vaccinia targets and 10 $TCID_{50}$ per cell for H1N1 targets. Spontaneous release was less than 15% for all groups. The effector cell/target cell ratio was 30:1.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 Induction of Anti-H1 Antibody

Mice were immunized as described in the methods section above and tested for the presence of IgG and IgA antibodies to H1. Titers of detected antibodies from each immunization group are shown in Table 1. Naive mice had no detectable anti-H1 antibody. Convalescent mice developed high levels of serum IgG, nasal IgA, and gut IgA anti-H1 antibody. Control mice immunized intraperitoneally with inactivated vaccine developed the highest titers of anti-influenza serum IgG antibody. Mice immunized with the vac/H1 recombinant by either scarification or intravenous injection developed high titers of anti-influenza serum IgG antibody, similar to those seen in convalescent mice, although not quite as high as seen in mice immunized with standard vaccine. Mice immunized intranasally and intrajejunally developed lower but readily detectable levels of serum IgG antibody. Intragastric immunization produced inconsistent results: four of six mice responded similarly to the intranasal and intrajejunal groups, but two were unresponsive.

Significant levels of mucosal (nasal wash and gut wash) IgA were induced only by influenza virus infection or by vac/H1 given by a mucosal route, i.e., into the nose, stomach, or jejunum. Intranasal, intrajejunal, and intragastric (four of six) immunized mice all had nasal wash titers similar to those of convalescent mice. Intrajejunal immunization produced gut wash titers similar to those of convalescent mice and higher than those of the intranasal or responding intragastric immunized mice. The administration of cimetidine, a drug that inhibits gastric acid secretion, did not increase the percentage of mice that responded to intragastric immunization. Neither parenteral route of immunization with vac/H1 (scarification or i.v.) nor i.p. immunization with the standard vaccine stimulated significant titers of mucosal IgA antibody (nasal wash IgA anti-H1 titers of 0.04, < 0.01 and 4 respectively).

Table 1. Anti-H1 influenza antibody titers by ELISA

GROUP	Serum IgG ($x10^{-3}$)	Nasal Wash IgA ($x10^{-5}$)	Gut Wash IgA ($x10^{-5}$)
Naive	<0.1 " 0.06	<0.01 " 0.01	<0.01 " 0.01
Convalescent	212 " 63	27 " 6	18 " 5
Standard vaccine - IP	406 " 159	4 " 2	2 " 1
vac/H1 - scarification[1]	123 " 32	0.07 " 0.06	0.2 " 0.06
vac/H1 - IV	187 " 38	<0.01 " 0.01	<0.01 " 0.01
vac/H1 - intranasal	24 " 7	36 " 11	12 " 4
vac/H1 - intrajejunal	22 " 1	27 " 7	19 " 3
vac/H1 - intragastric responders (n=4)[2]	21 " 6	25 " 8	15 " 3
vac/H1 - intragastric nonresponders (n=2)	< 0.1 " 0.01	< 0.01 " 0.001	< 0.01 " 0.001

[1]Two of six mice tails scratched with the vac/H1 developed low titers of both nasal and gut wash IgA. This is most likely due to auto- or cross-immunization from grooming (Meitin *et al.*, 1993).

[2]Mice that were immunized by the intragastric route were divided into two groups. One group (n=4) had similar serum IgG and mucosal IgA antibody titers to those seen with intranasal and intrajejunal immunization, while the second group (n=2) failed to develop any detectable anti-influenza antibody (< $0.01x10^{-3}$). (Statistically significant differences were not seen due to the low numbers of animals in the subdivided intragastric groups, i.e., 4 and 2.)

Example 2 - Induction of IgA Antibody and Cell-Mediated Immune Response

In a separate experiment, mice were immunized by either the intrajejunal or intragastric route described in the methods section. Six weeks later, vaginal wash fluid was obtained (Wu *et al.*, 1993). No detectable IgA anti-H1 antibody was present in the vaginal wash of naive mice. Intrajejunal immunization was significantly more successful than intragastric in inducing vaginal anti-H1 IgA antibody: 14/17 (82%) of mice vaccinated intrajejunally had detectable IgA (titer of 14 responding animals = .04 x 10^{-5} " .03 x 10^{-5}),

compared to 13/30 (43%) ($p < 0.05$, two-tailed Fisher's exact test) vaccinated intragastrically (titer of 13 responding animals = $.10 \times 10^{-5}$ " $.05 \times 10^{-5}$). This confirms that immunization of one mucosal surface will lead to immunization of others.

Memory CTL activity was determined for six of the intrajejunally immunized mice in two separate experiments as shown in Table 2. Mice immunized with wild-type vaccinia or naive mice had minimal activity. Mice convalescent from an influenza H1N1 infection yielded 32% lysis against vac/H1-sensitized targets, 49% against vaccinia containing the nucleoprotein gene (vac/NP)-sensitized targets, and 24% of lysis against H1N1-sensitized targets in the first experiment and 58% lysis against vac/H1 targets and 45% against vac/NP targets in the second experiment.

The mice immunized intrajejunally with vac/H1 had 32% lysis against vac/H1-sensitized targets. 11% lysis against vac/NP-sensitized targets. and 18% against H1N1-sensitized targets in the first experiment and 42% lysis against vac/H1 and 16% lysis against vac/NP in the second experiment. In another separate experiment, lysis of H3N2-sensitized P815 cells by splenocytes from vac/H1-immunized mice was less than 25% compared to 86% for lysis from a H1N1-infected mouse. Thus, most of the CTL activity was directed against the hemagglutinin and not to the vaccinia. The low anti-vaccinia CTL activity was undoubtedly due to the secondary *in vitro* stimulation with H1N1-sensitized stimulator cells rather than vaccinia-sensitized stimulator cells.

Table 2. Memory CTL activity induced by intrajejunal immunization with vac/H1.

Experiment	Mean % specific lysis of P815 cells sensitized with:			
	Group	vac/H1	vac/NP	H1N1
#1	Wild-type Vaccinia	3	7	9
	H1N1-infected	32	49	24
	vac/H1-intrajejunal	32	11	18
#2	Naive	1	1	n.d.
	H1N1-infected	58	45	n.d.
	vac/H1-intrajejunal	42	16	n.d.

Example 3 - Influenza Virus Challenge

In a third experiment, groups of mice that were immunized as described in the methods section above were challenged six weeks later with 20 MID_{50} of live influenza A PR8 (H1N1) administered intranasally while anesthetized (Yetter *et al.*, 1980). Mice were sacrificed 1, 3, 5, and 7 days after challenge and virus titers determined for nasal and lung tissues (Figure 1). The mice convalescent from a previous influenza infection had no virus in their noses. Naive mice had high titers of virus in their noses throughout the 7 days. Significantly lower levels of nasal virus were found in the three groups receiving successful mucosal immunization, i.e.. vac/H1 intranasal. vac/H1 intrajejunal, and vac/H1 intragastric responders. Four groups of mice (those immunized by standard vaccine i.p., vac/H1 scarification. vac/H1 i.v.. and vac/H1 intragastric nonresponders) had Day 1 nasal virus titers that were statistically indistinguishable from naive mice. Viral clearance after Day 1 was more rapid in mice that had received vac/H1 than either the naive mice. the non-responders, or those given standard vaccine i.p. Because respiratory viruses are primarily cleared by cytotoxic T-cells. this more rapid clearance is most likely due to the higher levels of anti-influenza CTL activity induced by vac/H1 than by standard vaccine (Bender *et al.*. 1990).

Virus titers in the lungs are also shown in Figure 1. Convalescent mice were solidly immune as evidenced by the lack of virus. Naive mice had high titers of virus throughout the seven days studied. The gastric non-responders were not statistically different from the naive mice in regard to virus shedding. All the remaining groups had statistically significantly ($p<0.05$) lower amounts of virus than the naive controls.

The variability in the amount of virus recovered from the intragastrically immunized mice (Figure 1) was similar to that seen in the intragastric group studied for antibody titer (Table 1). Fifteen of the 24 mice (62%) had reduced amounts of virus in the lungs and nose. The pattern was similar to that seen with other mucosally immunized animals. Mean serum IgG and gut wash IgA anti-influenza antibody titers measured in these same fifteen animals were 27 and 26 respectively, i.e.. an antibody titer similar to other mucosally immunized animals. The lung and nasal virus shedding patterns of the remaining nine mice were similar to previously uninfected controls, and these mice had no detectable serum IgG or gut wash IgA antibody.

Example 4 - Vaccination Against HIV Infection

Live recombinant vaccinia virus is prepared containing heterologous DNA sequences that encode various HIV antigens. For example, the HIV gene sequences can include those that encode GP160, GP120, or subunits of these or other HIV proteins. The live recombinants are then prepared in an enterically-coated dosage form. The host animal or human ingests the vaccine which dissolves upon reaching the host's intestine. In the intestine, the free virus replicates and induces a host immune response against the HIV expression products. Subsequent administrations of vaccine can be determined and administered when necessary.

Example 5 - Vaccination Against Hepatitis B

Live recombinant vaccinia virus is prepared containing heterologous DNA sequences that encode hepatitis B antigens. The live recombinants are then prepared in an enterically-coated dosage form. The animal or human to be vaccinated orally ingests the vaccine preparation, which dissolves upon reaching the host's intestine and releases free virus. In the intestine, the free recombinant virus replicates and induces a host immune response against the hepatitis B surface antigen expression products of the heterologous genes. Upon subsequent exposure to hepatitis B virus, the immunized host can produce a strong, multi-level immune response against hepatitis B which protects the host from infection with the virus. Optimal dosage levels and subsequent administration of the recombinant vaccine can be determined by those skilled in the art.

Example 6

Female BALB/c mice were immunized intragastrically (i.g.) with MVA HA-NP one hour after co-administration of cimetidine to inhibit gastric acid secretion and cholecystokinin to induce emptying of the gallbladder prior to vaccine administration. Positive control mice were convalescent from an H1N1 (influenza A/PR/8/34) infection or given MVA HA-NP i.m. Negative control mice were naive animals or mice given wild-type MVA i.m.

Female BALB/c mice, 5-6 weeks of age (Taconic Laboratories, Germantown, NY), were housed in specific pathogen free conditions. One hour before i.g. inoculations, each mouse received a combination of 3 mg of cimetidine HCl (SmithKline Beecham, Philadelphia, PA) and 0.02 Fg of sincalide (the C-terminal octapeptide of cholecystokinin)

(Squibb, Princeton, NJ) in 100 Fl of PBS i.p. Mice received 200 Fl of MVA HA-NP in 50 mM HEPES buffer (Mediatech, Washington, D.C.) containing 10^8 p.f.u. via a 1" (2.5 cm) feeding needle. In some mice, i.g. inoculation was repeated five weeks later. For i.m. inoculations, 100 Fl of wild-type MVA or MVA HA-NP, containing 0.5 X 10^8 p.f.u., was injected into each quadriceps muscle. Stock influenza viruses used for i.n. inoculation or challenge were grown in the allantoic cavity of 10-day old embryonated chicken eggs for 3 days at 35E C, harvested, and clarified. Virus stocks were titered on Madin-Darby canine kidney cells (Bender, 1992). Mice used as convalescent controls were infected i.n. with 20Fl of influenza A/Puerto Rico/8/34 (H1N1) containing $10^{7.1}$ $TCID_{50}$. For challenge studies at nine weeks post initial immunization, mice were anesthetized with sodium pentobarbital and inoculated i.n. with H1N1.

Oral vaccination was repeated in some of the animals five weeks later. Serum anti-H1 antibody was found in all mice receiving MVA HA-NP (either i.m. or i.g.) or H1N1 influenza virus (Figure 2A), though levels were lower in the i.g. immunized mice. Mucosal anti-H1 IgA antibody was detected in the vaginal secretions of 31 of 33 mice that received MVA HA-NP i.g. and in 9 of 9 positive controls convalescent from an H1N1 infection. At 8 weeks, vaginal IgA anti-H1 titers of mice which received two does of MVA HA-NP were about half the titer of mice convalescent from influenza (Figure 2B). The specific site of induction of mucosal IgA antibodies is unknown but may be due to abortive infection of the epithelial cells of the small intestine with MVA HA-NP. Direct surgical implantation of a replicating recombinant vaccinia virus into the jejunum was more immunogenic than i.g. administration (Meitin, 1994, *supra*) probably because bile and gastric acid can inactivate vaccinia virus (Meitin, 1991, *supra*). We determined the effect of pretreatment of mice with cimetidine and cholecystokinin and found that this pretreatment enhanced the proportion of animals responding to i.g. administration of MVA HA-NP from .60% to .100%. The appearance of anti-influenza IgA in vaginal and nasal wash following i.g. administration is consistent with the induction of the immune response in the intestine and migration of the immunocytes to other mucosal sites, a phenomenon recognized as part of the common mucosal immune system (McGhee *et al.*, 1990; McGhee *et al.*, 1992; Brandtzeag, 1989).

Example 7

To evaluate the functional significance of the antibodies induced according to Example 6, vaccinated mice with a wide range of antibody titers were chosen for challenge

with H1N1 influenza virus. Following this challenge, influenza virus reached a peak titer on day 3 post challenge of .10^4 TCID ^{50} in the noses of naive healthy mice (Figure 3A). The noses were protected best in those mice receiving two i.g. doses of MVA HA-NP (Figure 3A). Five of the 12 mice receiving two i.g. doses of MVA HA-NP shed no virus from their noses on day 1 post-challenge and the mean titer of the total group (0.65"0.2) was significantly lower than naive (1.9"0.4), MVA HA-NP i.m. (1.4"0.1), or MVA i.m. (1.6"0.1) mice. All five mice immunized twice with MVA HA-NP i.g. that were sacrificed three days post challenge had high pre-challenge vaginal IgA antibody titers and shed no virus from their noses. Protection of the MVA HA-NP i.g.-immunized mice correlated strongly with vaginal wash anti-HA titer; 6 of 6 mice with a titer # 1/8 shed virus as compared to 1 of 11 mice with a titer $ 1/16 (p=0.0004, Fisher's). As expected from the low anti-H1 IgA titers induced by one i.g. dose of MVA HA-NP. the noses of these mice were not protected from H1N1 challenge (1.5"0.2). Pulmonary virus shedding peaked at .10^5 TCID $_{50}$ on day 1 post-challenge (Figure 3B). Two i.g. doses of MVA HA-NP completely protected the lungs of 12 of 12 mice on the day following the challenge (Figure 3B) as did MVA HA-NP i.m. in 4 of 4 mice. One i.g. dose provided partial, but significant, protection on day one following challenge (1.2"0.4; mean" SE) as compared to naive (4.8"0.4) or MVA i.m. (4.6"0.3) mice.

Example 8

For determination of anti-influenza CTL activity, spleens were removed and cultured *in vitro* with H3N2-infected autologous splenocytes for 7 days and cytotoxicity was assayed against H3N2-sensitized P815 cells (Bender *et al.*, 1991, Bender *et al.*, 1993). Table 3 demonstrates that two i.g. doses of MVA HA-NP induced lower levels of anti-influenza CTL activity than did nasal H1N1 influenza virus. As a measure of the efficacy of this cell-mediated immune response, MVA HA-NP immunized mice were challenged with influenza A/Port Chalmers/1/73 (H3N2). As these mice did not have protective antibodies. initial (day 1) virus titers of the nose and lung were statistically indistinguishable in the immunized and control mice. Recovery of mice that had been immunized with two i.g. doses of MVA HA-NP was seen in nasal titers (Figure 4A). Lung virus titers were significantly lower than either naive mice or mice given MVA i.m. (Figure 4B). MVA HA-NP i.g. immunized mice had titers that were significantly lower than MVA-HA-NP i.m. immunized mice.

Table 3. Splenic cytotoxic T-lymphocyte activity of mice convalescent from H1N1 infection or vaccinated i.g. with MVA HA-NP*. Data are mean %^{51}Cr release from H3N2-sensitized P815 cells at two different effector:target ratios. Negative controls showed <5% lysis.

Mean % lysis of H3N2-sensitized P815 cells by mice inoculated with:			
H1N1 intranasal		MVA HA-NP i.g. x 2	
30:1	10:1	30:1	10:1
60	59	23	14
59	57	21	17
13	16	12	12

* Spleens were obtained from BALB/c (H-2d) mice two weeks post inoculation with 10^8 pfu of MVA HA-NP, PR8 infection, or wild-type MVA; cultured for 7 days with H3N2-sensitized autologous splenocytes; and tested in a 6-hr ^{51}Cr release assay against H3N2-sensitized P815 (H-2d) cells [Bender *et al.*, 1991; Bender *et al.*, 1993]. Percent specific lysis was determined as [(experimental release-spontaneous release)/(total release-spontaneous release)] X 100. Spontaneous release was < 10% of total.

Example 9

Experiments to establish the safety of enteric immunization with MVA/H1+NP.

We inoculated nude (athymic or T-cell deficient) mice with 10^8 pfu of wild-type vaccinia (VAC), vaccinia expressing the influenza hemagglutinin (VAC/HA), or vaccinia expressing the influenza nucleoprotein (VAC/NP) by either the intraperitoneal (i.p.) or intragastric (i.g.) route (n=5-6/group). Our results (Figure 5 below; the animals were followed for 60 days, but only the first 21 days are depicted, as no change occurred thereafter) demonstrate that:

1. As previously reported (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987) parenteral inoculation of nude mice with VAC or VAC/HA killed the mice within one week.
2. I.g. inoculation is safer than i.p. inoculation. Death is delayed by approximately five days with i.g. inoculation with VAC and does not occur with i.g. inoculation with VAC/HA, perhaps due to some viral inactivation in the gut. This observation also may be related to the fact that even nude mice have γ–δ T-cells lining their gut.
3. Addition of the NP gene further attenuates the virulence of the recombinant vaccinia virus. VAC/NP did not kill the animals whether given by the i.p. or i.g. routes. To establish that the NP gene attenuated the vaccinia and did not induce some non-specific immune defense in these animals, we inoculated SCID (B- and T-cell deficient) mice with

either VAC/HA, VAC/NP, VAC/HA+NP, or a mixture of VAC/HA and VAC/NP. We found (data not shown) that the VAC/HA and VAC/HA plus VAC/NP animals died, with no deaths in the VAC/NP or VAC/HA+NP mice. This suggests that the NP gene somehow attenuates the virulence of vaccinia virus. (Both the HA and NP genes are in the thymidine kinase gene of the vaccinia.)

4. MVA/H1+NP is extremely safe. Neither MVA nor MVA/H1+NP killed any SCID (n=6/group) mice (Figure 6) as did VAC-HA. (Once again, i.g. took longer to kill the animals than i.p.) Further, the MVA/H1+NP mice lost less than 10% of their body weight (data not shown). As in Figure 5, the animals were actually followed for a total of 60 days. We also found no mortality or morbidity in four newborn BALB/c mice given MVA/H1+NP, while all three newborns given VAC died. Another group of mice was injected i.p. or i.g. with MVA and sacrificed between 3 and 48 hours later; no gross pathology was noted, no virus was recovered from any culture, and (H&E) histological examination showed no damage. In control mice inoculated with either VAC or VAC-HA, high titers ($10^3$to more than 10^5 pfu) of virus were isolated from brain, lung, liver, and spleen.

Example 10

Experiments to establish the dose-response and duration of intragastric administration of MVA/H1+NP.

In these experiments we determined the minimal effective dose, whether a third dose would boost immunity, and whether the immunity was durable. Young BALB/c mice were immunized i.g. with various doses of MVA/H1+NP at three-week intervals and blood and vaginal wash obtained for determination of anti-influenza serum IgG antibody and mucosal IgA antibody. Mean titers and numbers of responding mice are shown in Table 4 and Figure 7. (Mice were immunized at weeks 0, 3, and 6. Comparable mean values for six H1N1 convalescent mice were serum ELISA titer of 7,400 and IgA of 32; naïve or MVA immunized mice had values of <0.1 and <2).

Table 4. Enhanced serum and mucosal antibody response with three i.g. doses of MVA/H1+NP.

	Two i.g. inoculations				Three i.g. inoculations			
Inoculum	IgG Responders	Serum IgG titer	IgA Responders	Vaginal IgA titer	IgG responders	Serum IgG/G titer	IgA responders	Vaginal IgA titer
10^8 pfu	6/6	6350	5/6	15	12/12	73,700	12/12	23
10^7 pfu	9/11	24	7/11	11	10/11	5100	9/11	25
10^6 pfu	1/10	1.3	3/10	4	6/12	46	4/12	6
10^5 pfu	0/11	<0.1	4/11	3	0/12	<0.1	7/12	3

The table shows that three doses of 10^8 pfu of MVA/H1+NP are required to induce high levels of immunity in all animals. Further, mucosal IgA is more readily induced than serum IgG. Figure 7 shows that three doses of MVA/H1+NP i.g. induce peak serum antibody titers equivalent to three doses given i.m. or an infection with live H1N1 influenza virus. At one year, serum antibody levels were essentially identical in all groups except MVA/H1+NP i.g. x2, which was somewhat lower at all time-points sampled. High levels of vaginal anti-H1 IgA antibody were detected in only those mice given a mucosal inoculation with MVA/H1+NP or H1N1 virus. This IgA persisted in relatively high levels in only the MVA/H1+NP i.g. x3 and H1N1 infected mice. *Finally, we found that both the noses and lungs of mice immunized with MVA/H1+NP i.g. x2 or x3 were protected from an influenza challenge.*

Example 10

Immunization of Higher Mammals Using the Vaccine of this Invention:

In Table 5 below, we provide results in a large mammalian model (equine) of experiments that are similar to those conducted in mice. These data indicate that the vaccine of this invention may be successfully applied to larger mammals, including horses, pigs, sheep, cows, and primates, including humans. Although the antigens expressed by the vaccines used in these experiments were influenza virus related, these results make it predictable that similarly positive results can be expected in other animals and humans.

TABLE 5.

Group	Horse No.	SERUM			SECRETIONS		
		Influenza	Neutralizing	Antibody	Influenza	Neutralizing	Antibody
		Pre-inoculation*	Post-1st Inoculation**	Post-2nd inoculation†	Pre-inoculation	Post-1st inoculation	Post-2nd Inoculation
H1N1 Challenge	768	0	0	16	2	64	32
	775	4	128	64	8	32	64
	776	0	32	256	2	64	NA
	782	2	32	64	8	4	512
STD Vaccine	770	4	>1024	>1024	1	128	32
	777	2	512	>1024	4	16	32
	785	0	128	>1024	2	32	32
	788	8	>1024	>1024	1	256	256
MVA (O)	771	2	4	NA‡	1	2	NA
	778	0	2		2	2	
	779	0	4		2	1	
	780	0	1		1	2	
MVA(H1+NP) i.m.	767	0	>1024	NA	4	16	128
	769	128	>1024		2	4	128
	772	0	>1024		1	8	32
	773	0	>1024		2	2	32
MVA(H1+NP) i.n.	783	0	16	>1024	1	8	32
	784	1	8	>1024	1	2	64
	786	0	32	>1024	4	4	64
	787	2	2	>1024	2	2	8
MVA(H1+NP) i.j.	Buddy	0	NA	NA	4	64	NA
	Dusty	0			4	2	
	Iso	0			4	4	
	Mama	0	0	0	2	2	2

*Pre-inoculation samples obtained before initial challenge or immunization
** Post-1st inoculation samples obtained 3-4 weeks after 1st challenge or immunization
†Post 2nd inoculation samples obtained 3-4 weeks after 2nd challenge or immunization
‡Not available.

In addition to establishing the ability to induce serum and mucosal immune responses in large mammals using the instant vaccine, we were able to demonstrate that no viral particles could be recovered from equine blood (Table 6), feces (Table 7), or vaginal secretions (Table 8).

TABLE 6. Controls for the Isolation* of MVA(H1+NP) from Horse Blood

Method of inoculation	10^{1}**	10^{2}	10^{3}	10^{4}	10^{5}	10^{6}
1.0 onto monolayer	7†	142	TNTC	TNTC	TNTC	TNTC
0.1 into 2ml medium	5	45	577	TNTC	TNTC	TNTC
10μl into blood "Dusty"	0	0	0	17	419	TNTC
			Date			
Horse No.	Day1	Day 2	Day 3	Day 4	Day 5	Day 6
783‡	0†	0	0	0	0	0
784	0	0	0	0	0	0
786	0	0	0	0	0	0
787	0	0	0	0	0	0

*Isolation performed in chicken embryo fibroblast (CEF) cultures.
** Number of virus particles inoculated.
†Number of stained viral plaques or individual cells after 24 hours.
‡Undiluted blood (0.1 ml) from each horse [5/6/97-5/11/97] was inoculated onto susceptible CEF cultures and stained after 24 hours for MVA(H1+NP).
TNTC = too numerous to count.

TABLE 7. Controls for the Isolation* of MVA(H1+NP) from Horse Feces after Intrajejunal (i.j.) Inoculation

	10^9 ffu MVA(H1+NP) -70°C stock titration	Treated virus** 10^9 ffu MVA(H1+NP)	Negative feces with 10^9 ffu MVA(H1+NP)	Horse feces post-i.j. 10^9 ffu MVA(H1+NP)
-1	+	+	+	-
-2	+	+	-	-
-3	+	+	-	-
-4	+	+	-	-
-5	+	+	-	-
-6	+	+	-	-
-7	+	+	-	-
-8	+	-	-	-
Titer	$\geq 10^8$ ffu‡	$\geq 10^7$ ffu	$\geq 10^1$ ffu	$<10^1$ ffu

*Isolation performed in chicken embryo fibroblast (CEF) cultures, followed by immunostaining for MVA(H1+NP).

** 10^9 ffu of MVA(H1+NP) was inoculated into a 1:10 dilution of negative horse feces. Treatment of all diluted feces included incubation at 4°C for 1 hour, clarification by three centrifugations for 30 minutes at 3000rpm at 4°C, the ultracentrifugation of the supernatant at 150,000g for 2 hours at 4°C. The re-suspended pellet was inoculated onto CEF cultures.

†Intrajejunal (i.j.) horse inoculation with 1×10^9 ffu of MVA(H1+NP).

‡10^8 ffu of MVA(H1+NP) was titrated in 10-fold dilutions in CEF cultures and immunostained.

TABLE 8. Isolation* of MVA(H1+NP) from Nasal Swabs and Tampons

Horse Number	Sample Date	Days post-inoculation**	Result
783 784 786 787	4/22/97	1	Negative[†] ↓
783 784 786 787	4/23/97	2	Negative ↓
783 784 786 787	4/25/97	4	Negative ↓
783 784 786 787	4/26/97	5	ND Negative ↓
783 784 786 787	4/28/97	7	ND Negative ↓
783 784 786 787	4/29/97	8	Negative ↓
MVA(H1+NP) control	Stock titration	1×10^8 ffu [‡] inoculated	1×10^8 ffu recovered

*Isolation of MVA(H1+NP) done on chicken embryo fibroblast (CEF) cultures.
** Intranasal inoculation of 10^8 ffu of MVA(H1+NP).
[†]No stained plaques or individual cells were seen in undiluted cultures after immunostaining for the recombinant MVA(H1+NP).
[‡]1×10^8 ffu = 10^8 focus forming units of the same virus that was inoculated into horses was simultaneously titrated in CEF cultures to assess the sensitivity of the assay.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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Claims

1. A live recombinant vaccine comprising an heterologous polynucleotide molecule encoding an antigen for administration into a vertebrate in which said recombinant virus cannot replicate or replicates inefficiently. wherein said vaccine is administered intranasally, intragastrically, orally or intrajejunally, such that said vaccine contacts mucosal surfaces to induce mucosal as well as serum and cellular immune responses against said antigen.

2. The live recombinant vaccine of claim 1 which is live recombinant vaccinia, pox virus or vaccinia mutant vaccine capable of expressing a heterologous polynucleotide molecule in a host. wherein said vaccine is specifically adapted to be administered to the host in a manner to induce serum IgG antibody. mucosal IgA antibody. and cell-mediated immune responses directed against said heterologous polynucleotide molecule expression product, wherein said specific adaptation comprises provision of an enteric coating whereby the virus is released only when it reaches the host's small intestine. and wherein said vaccinia or poxvirus is replication deficient in mammals.

3. The vaccine, according to claim 1. wherein said heterologous polynucleotide molecule encodes at least one antigen expressed by an organism or virus that is pathogenic to the host.

4. The vaccine. according to claim 1. wherein said heterologous polynucleotide molecule comprises at least one polynucleotide molecule from more than one pathogenic organism or virus.

5. The vaccine, according to claim 1, wherein said recombinant vaccinia mutant is selected from the group consisting of MVA and canary pox virus.

6. A method for inducing a protective immune response in a host organism comprising immunizing a host with the vaccine according to claim 1, wherein said vaccine is enterically, intranasally or orally administered to the host.

7. A method for inducing a protective immune response in a host organism comprising immunizing a host with the vaccine according to claim 2.

8. A method for oral vaccine administration which comprises administering a replication deficient recombinant vaccine or pox virus to a host under circumstances that result in delivery of said vaccine to the small intestine such that the vaccine is protected from exposure to stomach acid or bile.

9. The method of claim 8 wherein said host is pre-treated, prior to said vaccine administration, such that, stomach acid and bile is limited at the time that said vaccine is administered orally or intragastrically.

10. The method of claim 9 for oral vaccine administration which comprises:

a) preparing a replication deficient recombinant vaccinia or pox virus;

b) pre-treating a host in need of vaccination with an acid-release blocker or a cholecystokinin antagonist or both prior to orally administering said recombinant virus; and

c) orally administering said recombinant virus.

11. The method of claim 10 wherein said virus is a recombinant MVA.

12. The method of claim 11 wherein said recombinant MVA comprises at least one influenza virus gene.

13. The method of claim 12 wherein said recombinant MVA contains an influenza virus hemagglutinin gene, an influenza virus nucleoprotein gene or both.

14. The method of claim 13 wherein said recombinant MVA is enterically coated.

15. A method for boosting the immune response against a pathogen or antigen in a geriatric mammal which comprises mucosal administration of a replication deficient live recombinant viral vector to said geriatric mammal wherein said recombinant viral vector comprises a polynucleotide encoding said antigen or an antigen of said pathogen.

16. The method of claim 15 wherein said antigen is an influenza virus antigen.

17. The method of claim 16 wherein said mammal is a horse, a cow, a sheep, a goat, a pig, or a primate.

18. The method of claim 17 wherein said primate is a human.

19. A method for inducing mucosal and serum immune responses against at least one antigen in a mammal wherein said immune responses persist for at least one year, said method comprising orally administering to said mammal at least two doses of a replication deficient poxvirus vector encoding said at least one antigen.

20. The method of claim 20 which comprises administration of at least three doses of said replication deficient poxvirus vector.

21. A method for immunizing immunodeficient mammals without causing mortality or morbidity which comprises administration of a live recombinant replication deficient viral vector.

22. A method for attenuating vaccinia virus which comprises insertion of the influenza NP gene into the vaccinia genome.

FIG. 1A

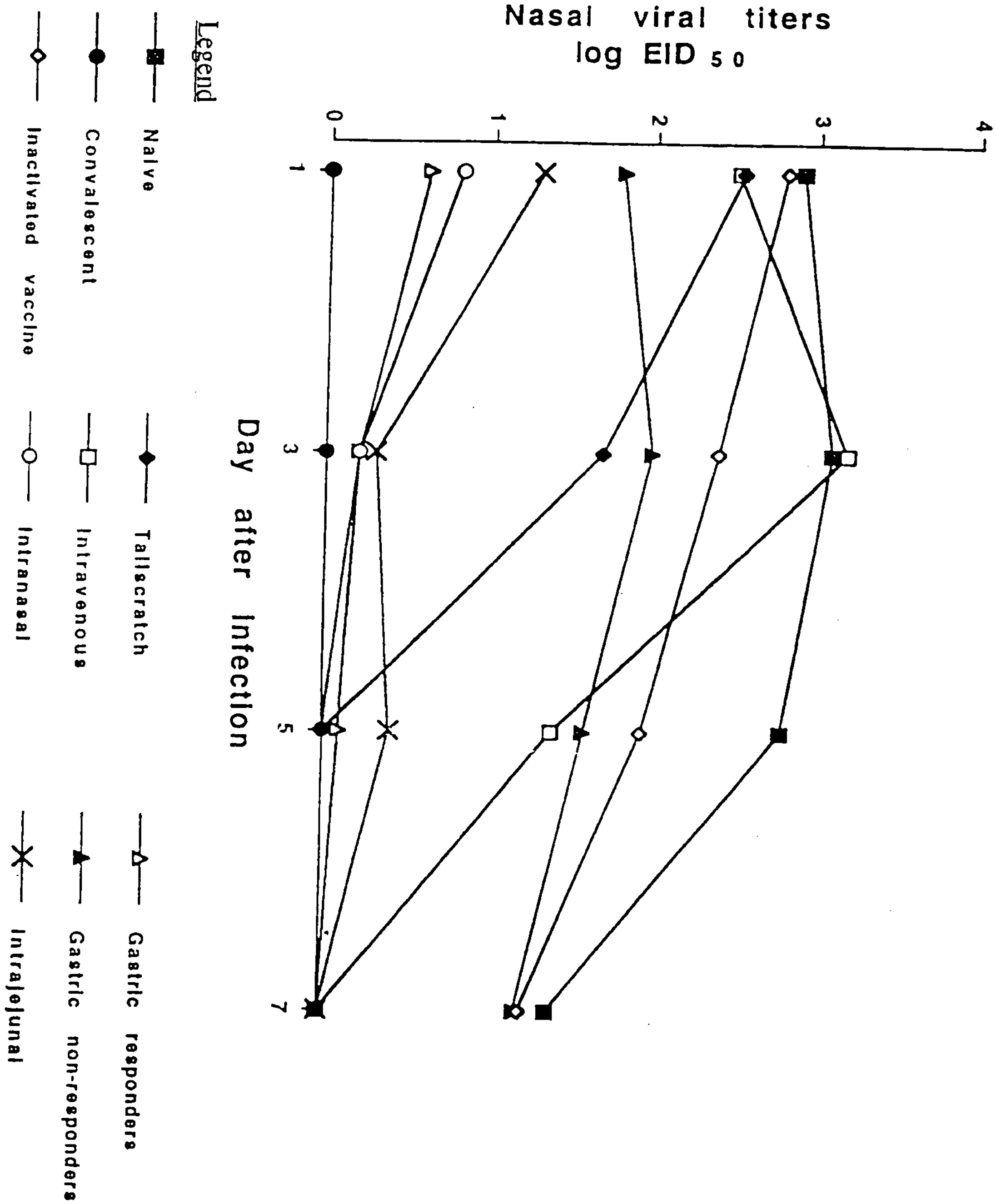
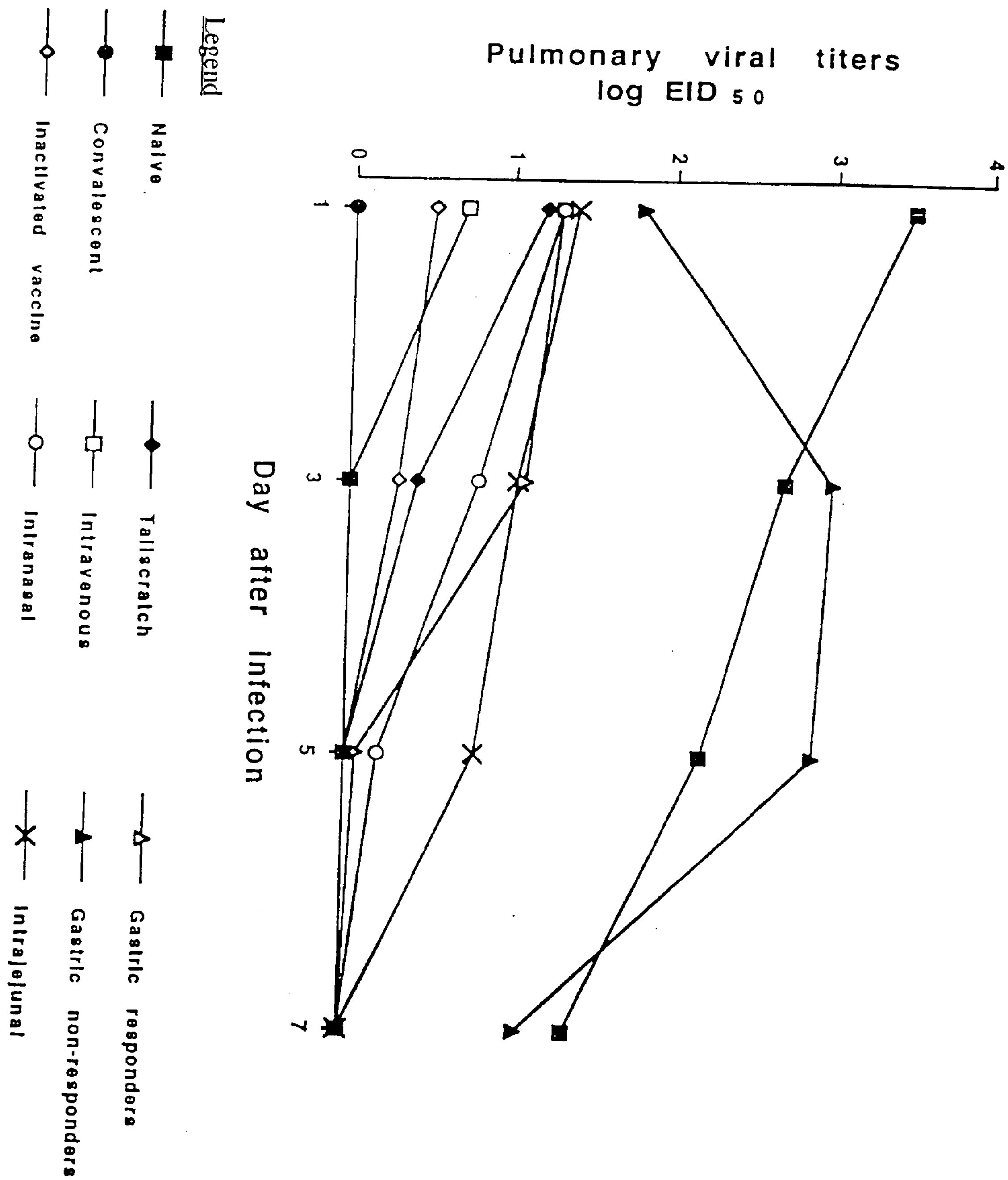

Nasal viral titers
log EID 50
0
1
2
3
4
1
3
5
7
Day after Infection
Legend
Naive
Convalescent
Inactivated vaccine
Tailscratch
Intravenous
Intranasal
Gastric responders
Gastric non-responders
Intrajejunal

FIG. 1B


Pulmonary viral titers
log EID 50
0
1
2
3
4
1
3
5
7
Day after infection
Legend
Naive
Convalescent
Inactivated vaccine
Tailscratch
Intravenous
Intranasal
Gastric responders
Gastric non-responders
Intrajejunal

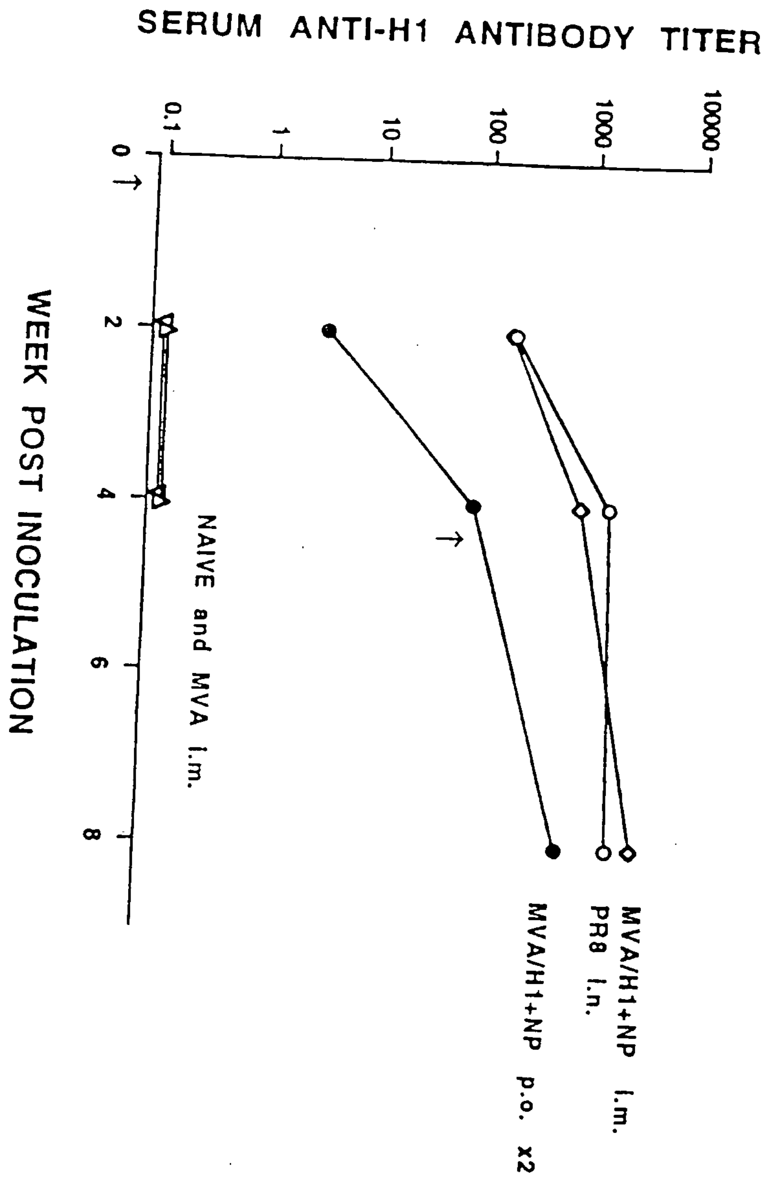

SERUM ANTI-H1 ANTIBODY TITER
10000
1000
100
10
1
0.1
0
2
4
6
8
WEEK POST INOCULATION
NAIVE and MVA i.m.
MVA/H1+NP i.m.
PR8 i.n.
MVA/H1+NP p.o. x2

FIG. 2A

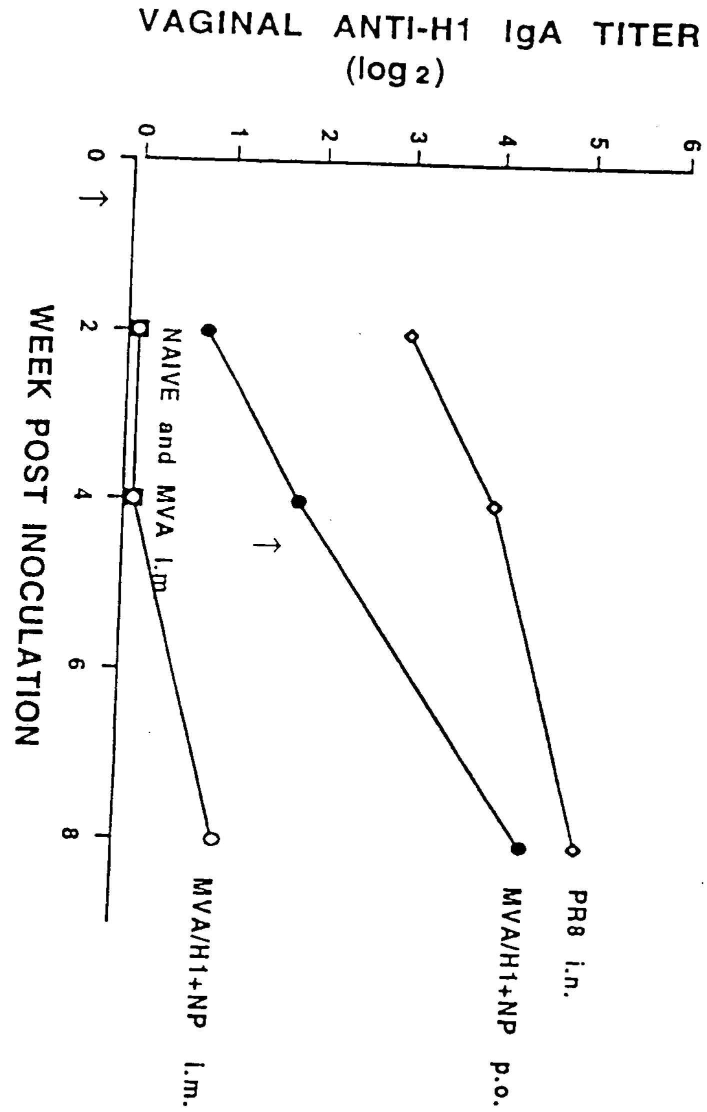

VAGINAL ANTI-H1 IgA TITER
(log 2)
0
1
2
3
4
5
6
0
2
4
6
8
WEEK POST INOCULATION
NAIVE and MVA i.m.
PR8 i.n.
MVA/H1+NP p.o. x2
MVA/H1+NP i.m.

FIG. 2B

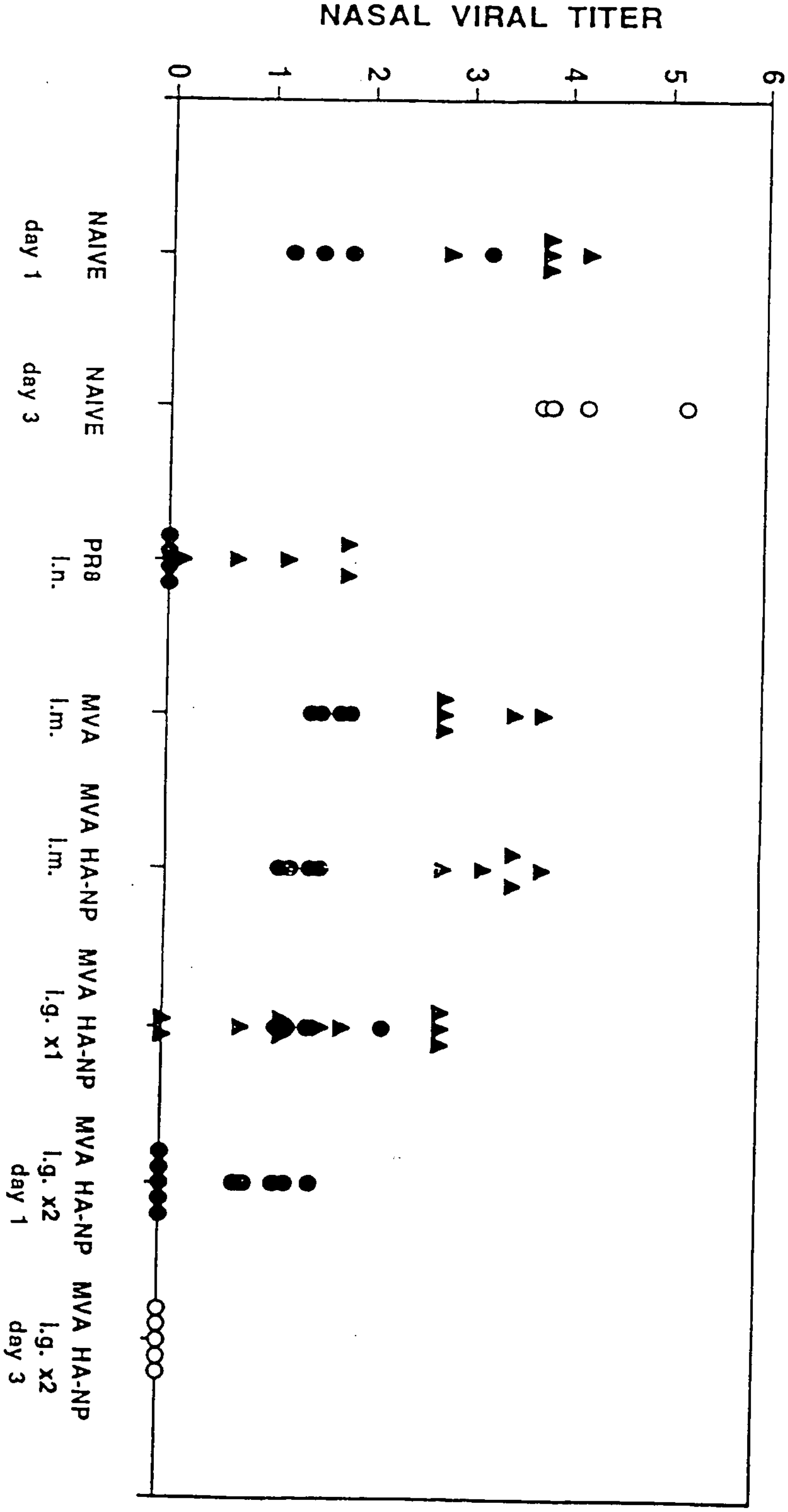

NASAL VIRAL TITER
0
1
2
3
4
5
6
NAIVE
day 1
NAIVE
day 3
PR8
i.n.
MVA
i.m.
MVA HA-NP
i.m.
MVA HA-NP
i.g. x1
MVA HA-NP
i.g. x2
day 1
MVA HA-NP
i.g. x2
day 3

FIG. 3A

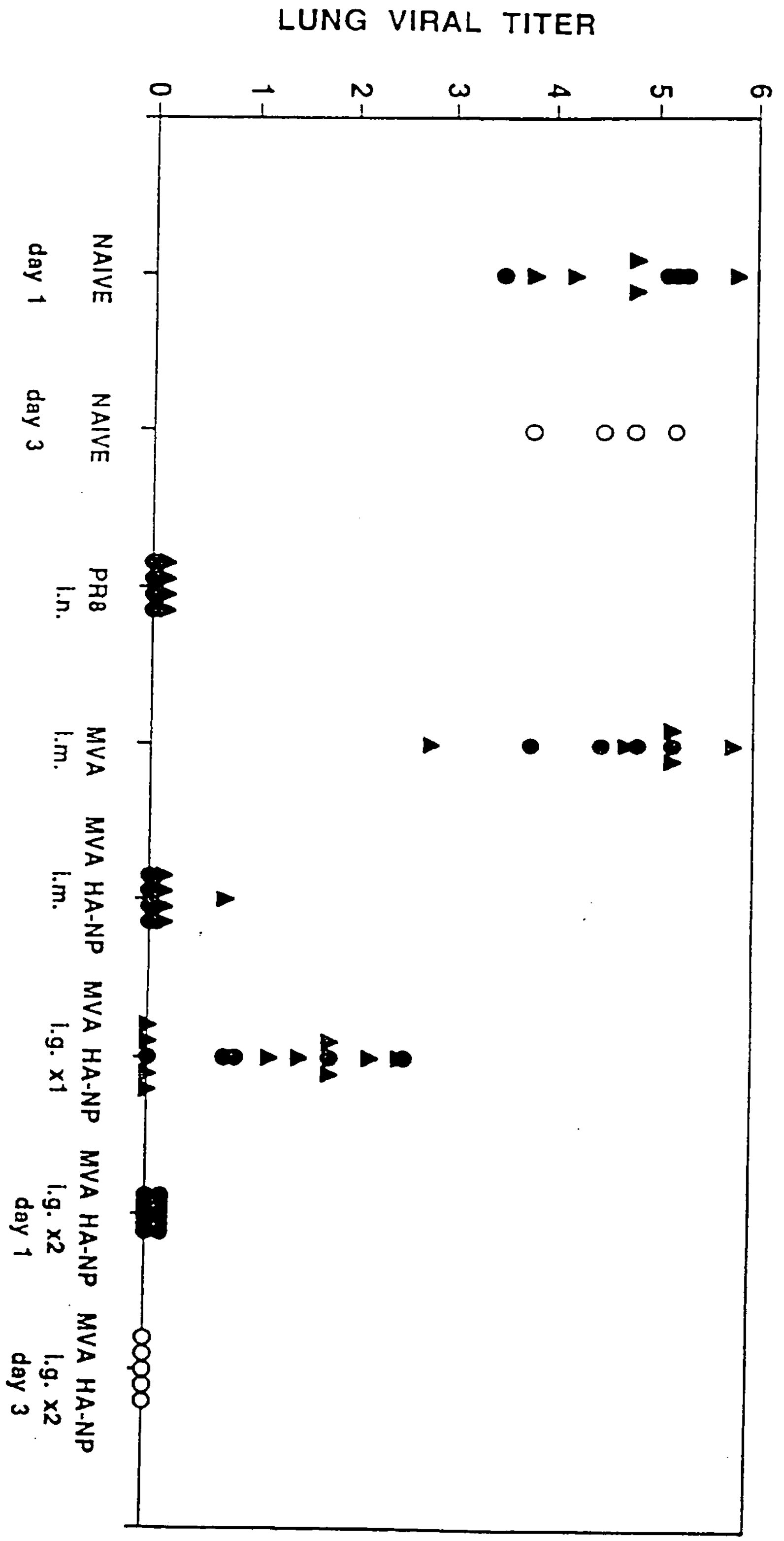

LUNG VIRAL TITER
0
1
2
3
4
5
6
NAIVE day 1
NAIVE day 3
PR8 i.n.
MVA i.m.
MVA HA-NP i.m.
MVA HA-NP i.g. x1
MVA HA-NP i.g. x2 day 1
MVA HA-NP i.g. x2 day 3

FIG. 3B

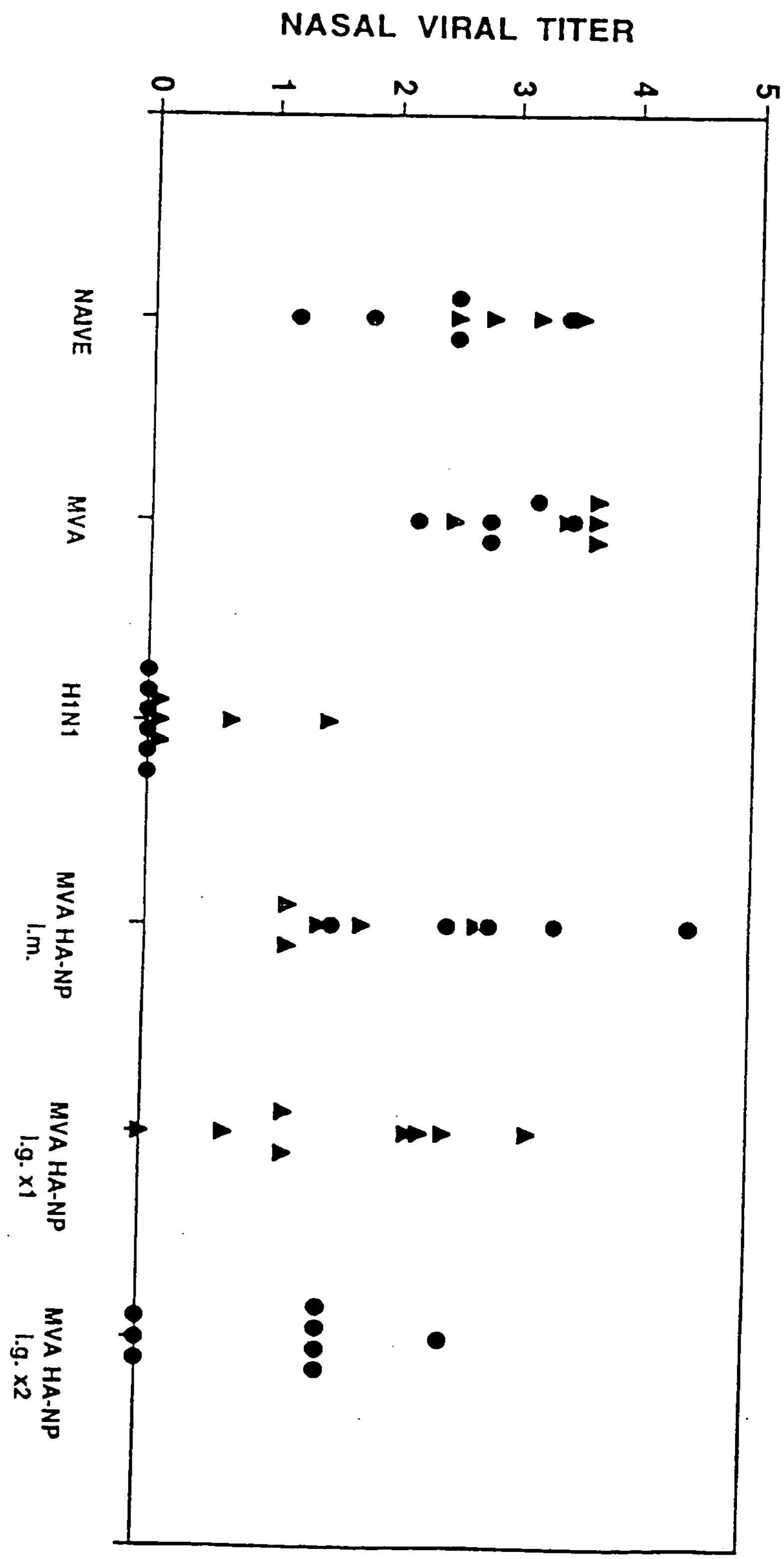

NASAL VIRAL TITER
0
1
2
3
4
5
NAIVE
MVA
H1N1
MVA HA-NP
i.m.
MVA HA-NP
i.g. x1
MVA HA-NP
i.g. x2

FIG. 4A

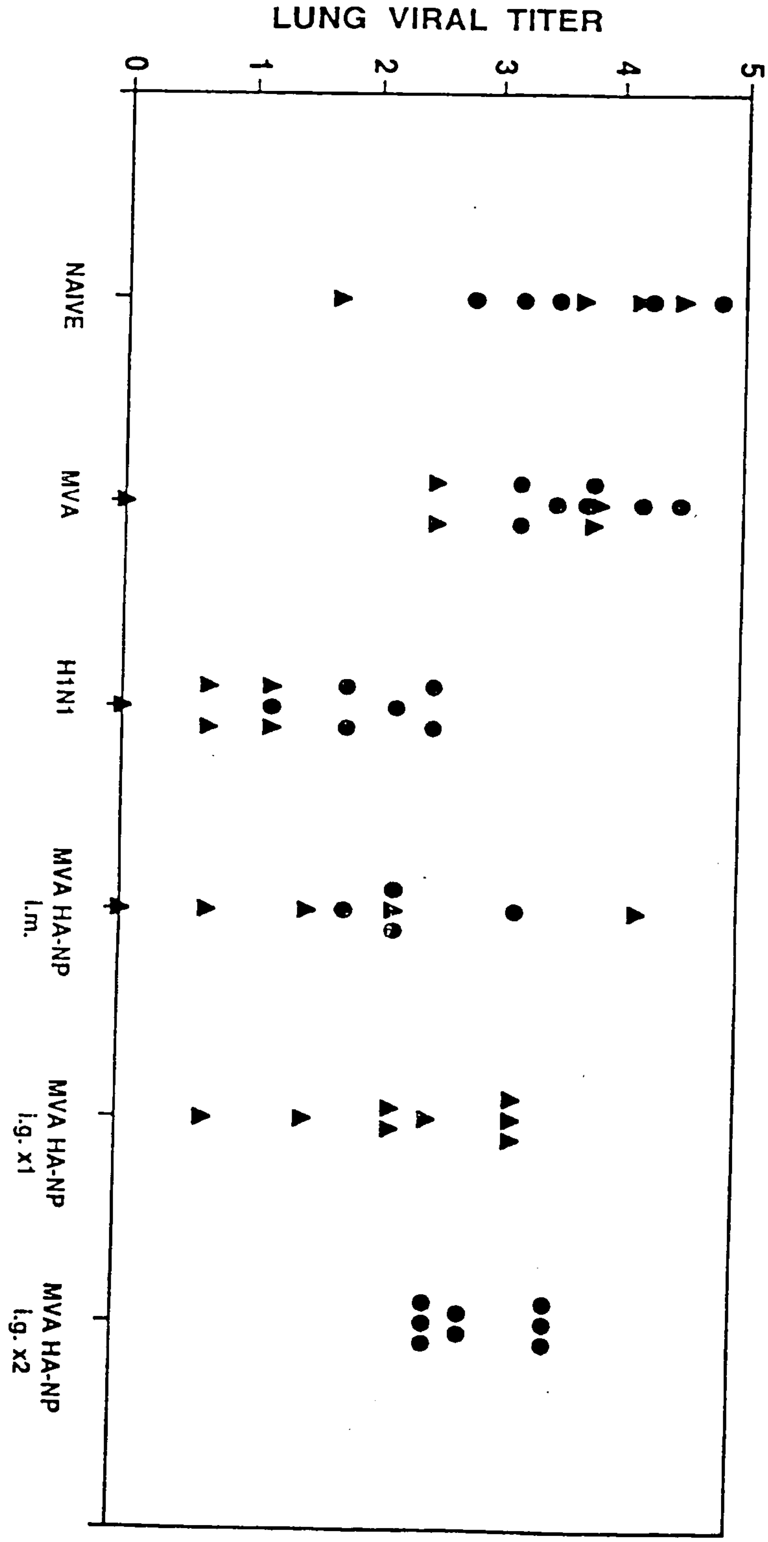

LUNG VIRAL TITER
0
1
2
3
4
5
NAIVE
MVA
H1N1
MVA HA-NP
i.m.
MVA HA-NP
i.g. x1
MVA HA-NP
i.g. x2

FIG. 4B

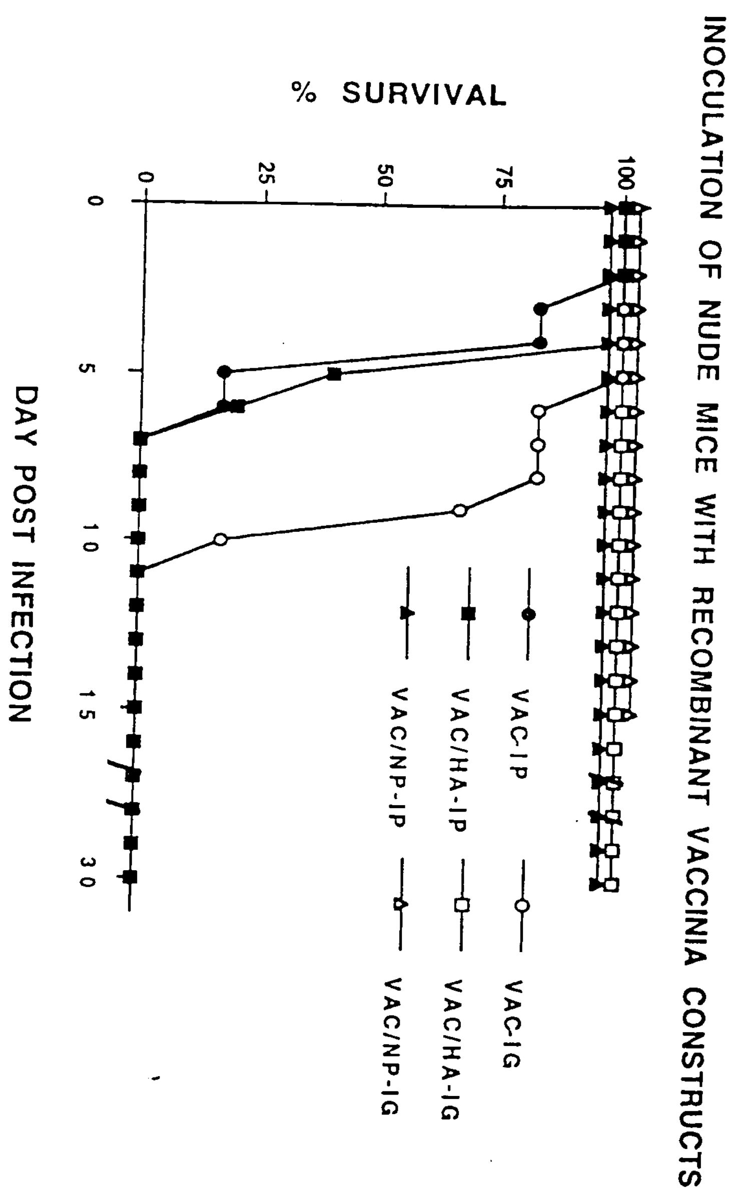

INOCULATION OF NUDE MICE WITH RECOMBINANT VACCINIA CONSTRUCTS
% SURVIVAL
100
75
50
25
0
0
5
10
15
30
DAY POST INFECTION
VAC-IP
VAC-IG
VAC/HA-IP
VAC/HA-IG
VAC/NP-IP
VAC/NP-IG

Figure 5

FIGURE 6

MVA HA-NP IS SAFE IN SCID MICE

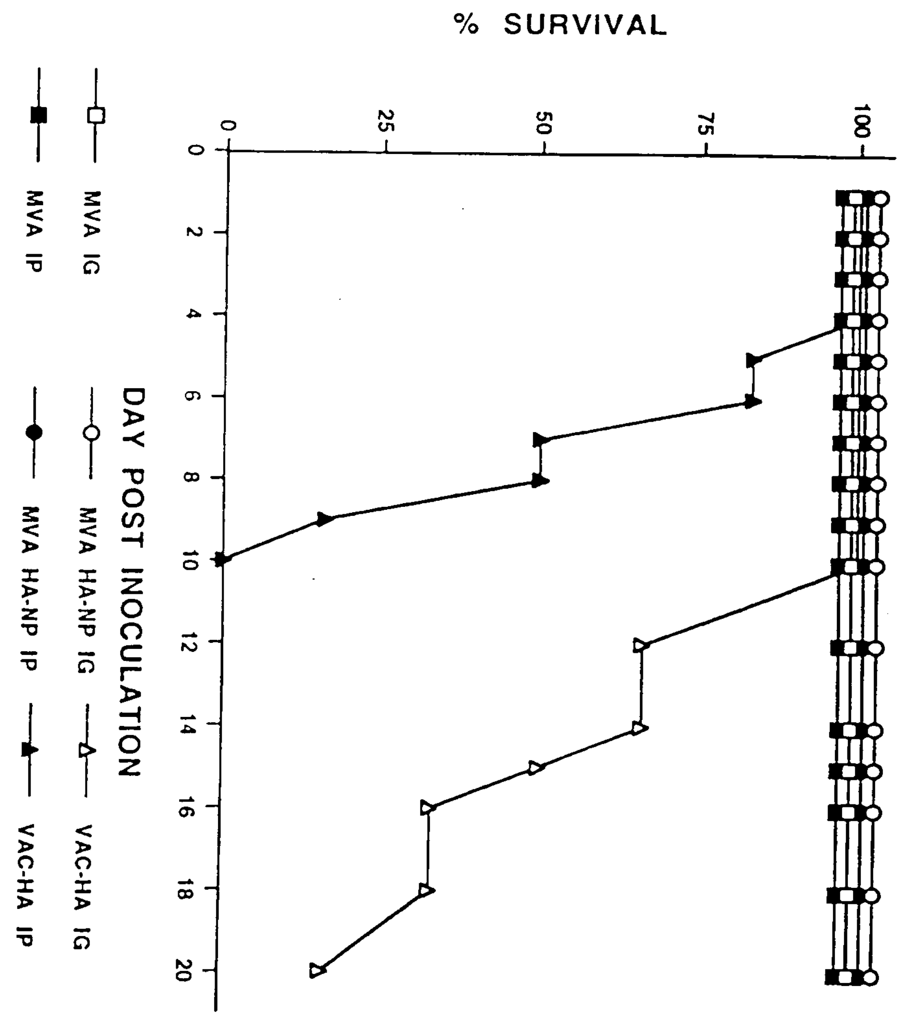
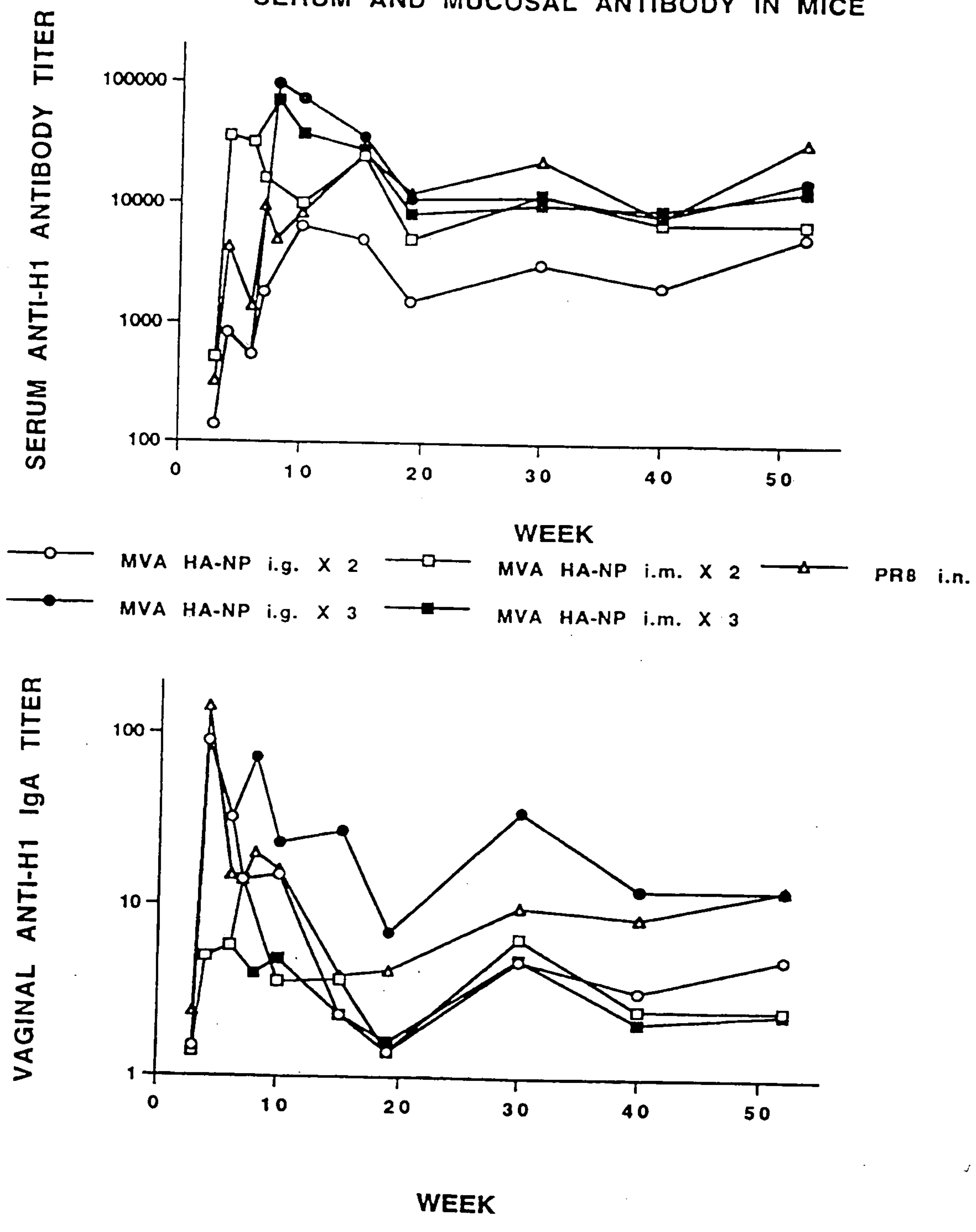


FIGURE 7.

THREE DOSES OF ORAL MVA HA-NP INDUCE LONG-TERM SERUM AND MUCOSAL ANTIBODY IN MICE



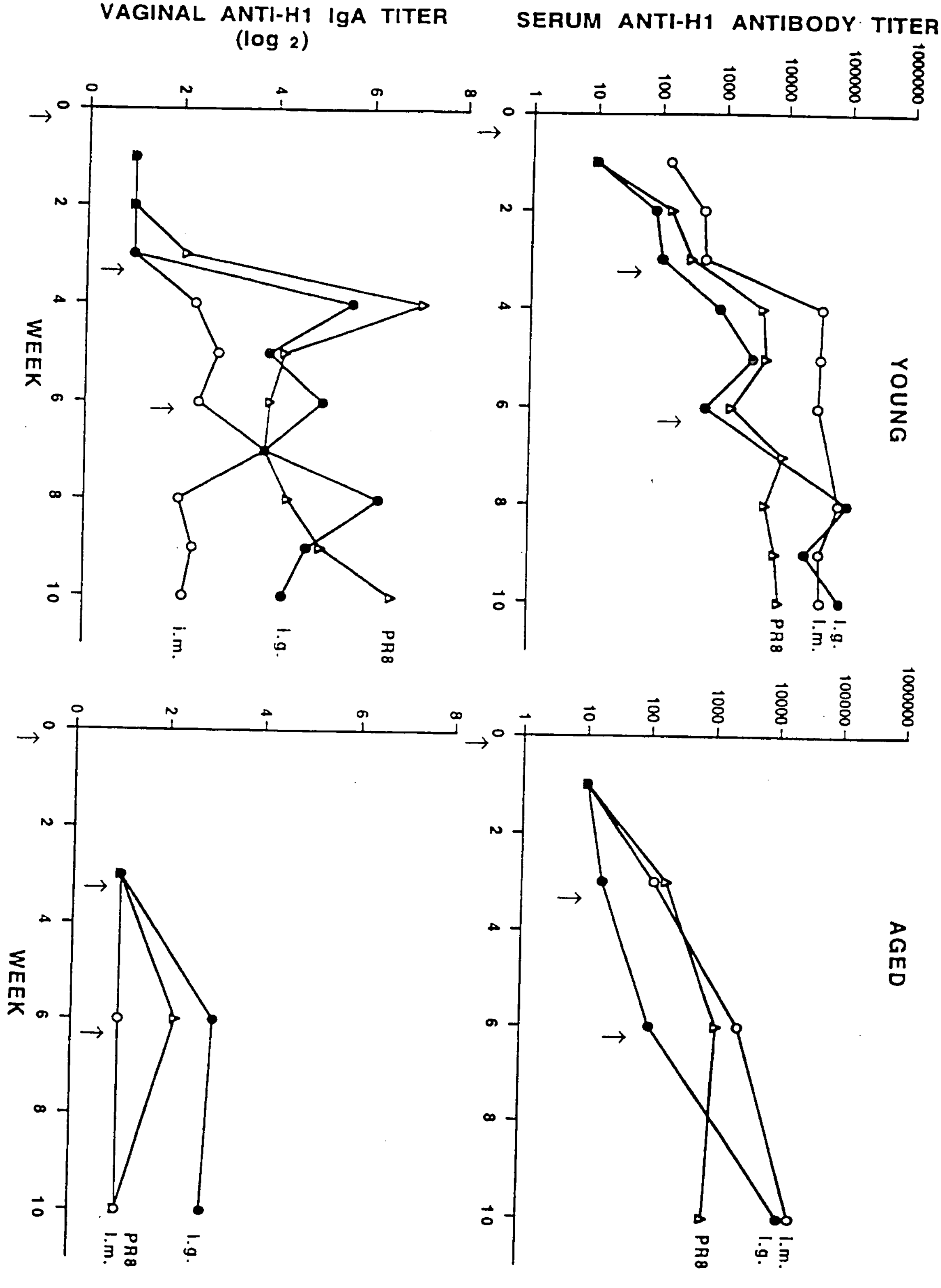
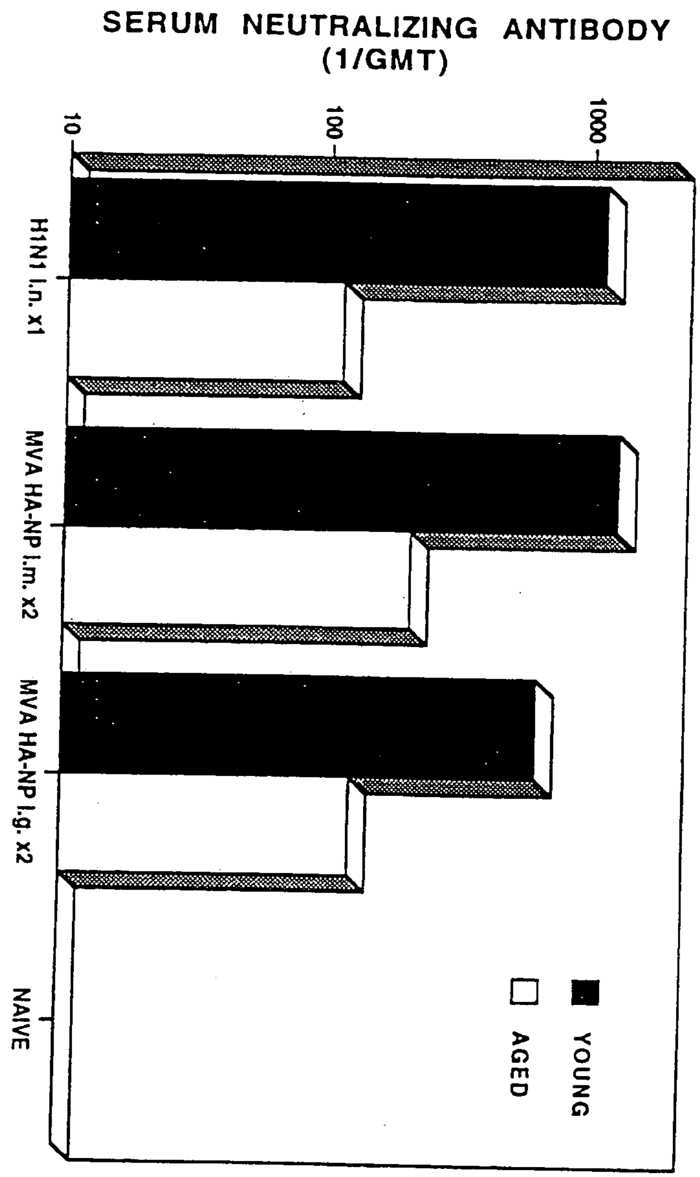


FIGURE 8. THREE DOSES OF ORAL MVA HA-NP VACCINE INDUCE SERUM AND MUCOSAL ANTIBODY IN YOUNG AND AGED MICE

FIGURE 9.

MVA HA-NP INDUCES NEUTRALIZING ANTIBODY IN YOUNG AND AGED MICE



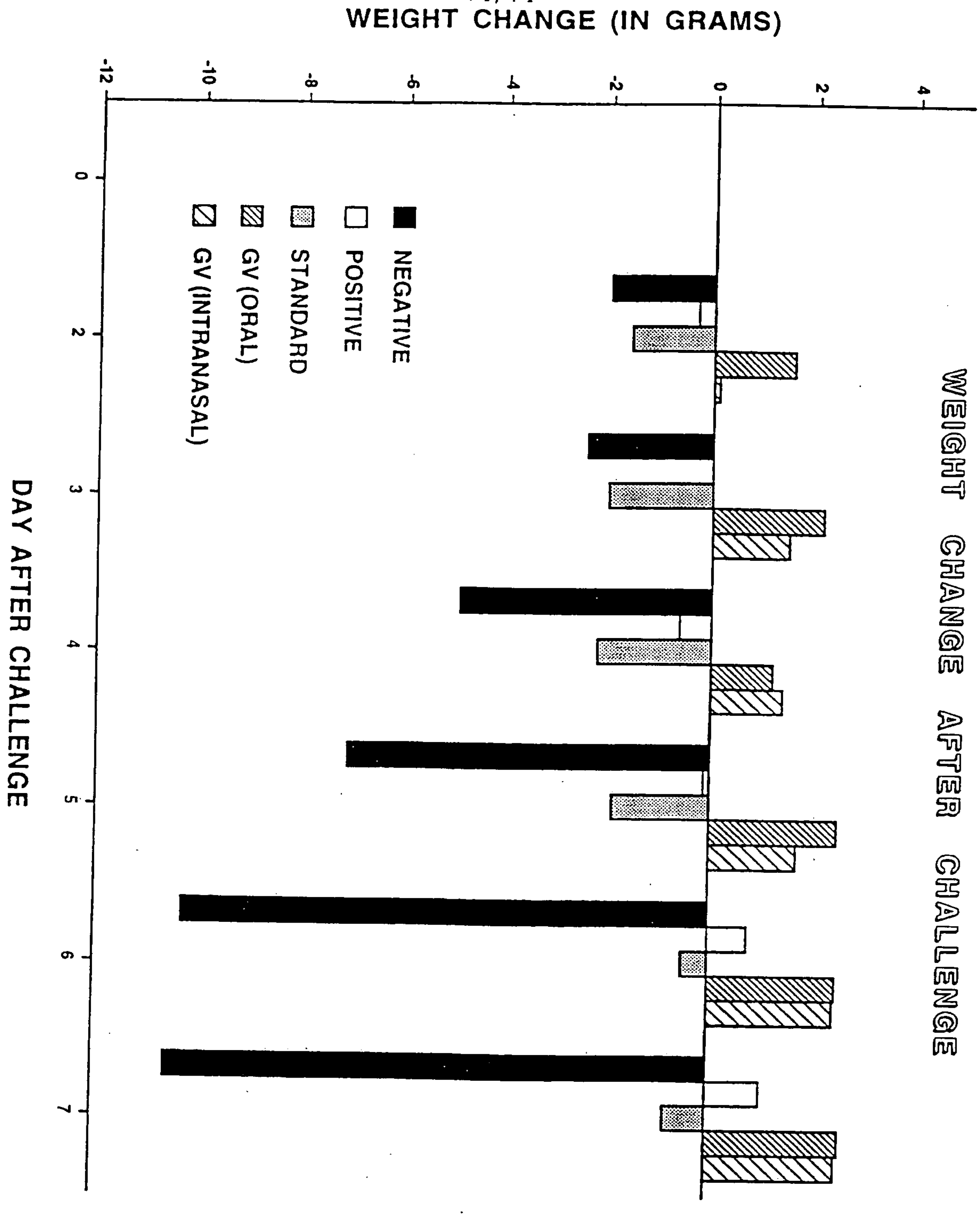

WEIGHT CHANGE (IN GRAMS)
WEIGHT CHANGE AFTER CHALLENGE
-12
-10
-8
-6
-4
-2
0
2
4
NEGATIVE
POSITIVE
STANDARD
GV (ORAL)
GV (INTRANASAL)
0
2
3
4
5
6
7
DAY AFTER CHALLENGE

FIGURE 10

Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty:

In regard to any state that is a signatory to the Patent Cooperation Treaty and which provides a twelve month grace period from the date of an applicant's or inventor's own disclosure, to the extent that any subject matter in this application may have been disclosed by Bender et al., in the Journal of Virology, 70(9):6418-6424, 1996, which became publicly available on August 6, 1996, it is hereby requested that such grace period be extended to this application. With respect to any new subject matter that is included (disclosed and claimed) in the present application, which new subject matter was not disclosed in the above referenced publication, it is respectfully urged that the referenced publication likewise be discounted for novelty of invention in all designated states.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13836

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K39/145 C12N7/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BENDER ET AL.: "Oral immunization with a replication-deficient recombinant vaccinia virus protects mice against Influenza" JOURNAL OF VIROLOGY, vol. 70, no. 9, September 1996, pages 6418-6424, XP002055082 see abstract see page 6418, column 2, last paragraph - page 6422, column 1, paragraph 1	1-14,19
Y		15-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 February 1998	27. 02. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Muller-Thomalla, K

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 89 03429 A (HEALTH RESEARCH INC.) 20 April 1989 see abstract see page 1, line 24 - page 2, line 13 see page 4, line 18 - page 5, line 36 see page 11, line 34 - page 13, line 24 see claims 1-39; examples 9,10	1-4,6,7
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X	BLANCOU ET AL.: "Oral Vaccination of the fox against rabies using a live recombinant vaccinia virus" NATURE, vol. 322, 1986, pages 373-375, XP002055040 see page 373, column 2, last paragraph - page 374, column 2, paragraph 1 see page 375, column 2, paragraph 4	1-4,6,7
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13836

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 1 333 512 A (STICKL HELMUT) 10 October 1973 cited in the application see page 2, line 6-125 see page 3, line 56 - page 4, line 80	
A	WO 92 16616 A (VIROGENETICS CORP.) 1 October 1992 see abstract see page 1, line 11 - page 4, line 3 see claims 1-26	

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